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CIRCULATING TUMOUR DNA IN PATIENTS WITH UNRESECTABLE PANCREATIC ADENOCARCINOMA

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"He drew a deep breath.
'Well, I'm back', he said."
J. R. R. Tolkien

A mia nonna, Teresina.

Index

Abstract

Pancreatic adenocarcinoma is the deadliest common malignancy. At the present time, it is the tumour causing the fourth greatest number of deaths in the United States and has been predicted to become the second top cancer killer by fifteen years' time. Despite recent advancements in medical treatment, the median survival for patients presenting with advanced pancreatic cancer, who represent approximately 75% of all patients, is no longer than 18 months for locally advanced disease and 12 months for metastatic cancer.

CA19.9 is the only approved tumour marker for pancreatic cancer. However, it is suboptimal in many respects, and, so as to allow optimal treatment of all patients with pancreatic adenocarcinoma, new biomarkers are urgently needed. An ideal marker could be used in many different phases of the management of this disease, such as detection, monitoring of minimal residual disease after resection, and assessment of chemotherapy efficacy.

The collection of a tissue specimen via biopsy is, at present, essential for the diagnosis of cancer and the molecular analysis of tumour genome. Obtaining a tissue sample in pancreatic cancer is extremely challenging due to anatomic and clinical reasons, and up to 30% of biopsies are inconclusive.

Cell-free DNA can be isolated from plasma in a variety of clinical scenarios. In oncologic patients, cancer-specific genetic alterations can be recognised in circulating DNA fragments. This indicates the presence of circulating tumour DNA (ctDNA) in the bloodstream, originating from apoptotic or necrotic cells in the primary tumour, metastases, or in the bloodstream. The detection, measurement and genetic characterisation of ctDNA may provide the clinician with a real-time genetic profile representative of all tumour lesions. The mini-invasiveness of this technique, which would only require a blood sample, allows serial monitoring of tumour dynamics. This approach has been called the "liquid biopsy"; in contrast, the conventional tissue biopsy only provides a snapshot of one tumour lesion at a single time point.

The presence of mutant sequences of the *KRAS* oncogene in the circulating cell-free DNA of a substantial proportion of patients with pancreatic adenocarcinoma has been highlighted in some studies to date. Recent works in other malignancies, such as colon cancer and lung cancer, suggest that the analysis of ctDNA may provide useful information as a diagnostic, predictive, and prognostic tumour marker.

In our proof-of-concept study, we investigated the possible role of ctDNA as a marker of response to treatment in a cohort of patients with locally advanced and metastatic pancreatic adenocarcinoma. Sixty-two blood samples were collected from 24 prospectively recruited patients before the initiation of chemotherapy (FOLFIRINOX or gemcitabine plus nab-paclitaxel), 15 days and 60 days after chemotherapy institution, and when imaging provided confirmation of disease progression. Droplet Digital PCR was used to detect *KRAS* mutations in plasma samples. Median follow-up time was 6.5 months.

Nineteen patients (79%) had detectable mutant *KRAS* in the baseline plasma sample. Three patients turned from *KRAS*-negative to *KRAS*-positive during the course of chemotherapy; circulating tumour DNA was, therefore, detectable in 22 patients (91%) throughout the follow-up, a higher proportion than in any other previous study.

Among the 22 patients with detectable circulating tumour DNA at any point of the follow-up, 20 provided more than one blood sample, thus allowing the evaluation of ctDNA concentration trends as indicators of response to treatment or progression. For 14 (70%) of these patients, ctDNA trends were concordant with imaging findings at 8-12 weeks of treatment. ctDNA levels remarkably declined, paralleling CA19.9 ones, in patients with partial response or stable disease. ctDNA increased almost four-fold in the group of patients who did not respond to chemotherapy and rapidly experienced progression, while the average CA19.9 level did not rise. In the subgroup of patients with disease control and concordantly downward ctDNA trend, a marked fall in ctDNA concentration (-91%) anticipated the radiological response by 54 days. In the group of patients with disease control, ctDNA levels declined on average by almost 50% from baseline to day 15; in

patients encountering disease progression during the follow-up, the same value increased on average by 250%.

Baseline ctDNA concentrations showed a moderate correlation with tumour burden, achieving a borderline level of statistical significance ($p = 0.06$), with progression-free survival ($p = 0.08$), and overall survival ($p = 0.06$).

Survival analysis did not show the progression-free survival to be statistically different for *KRAS*-positive and *KRAS*-negative patients at baseline. However, just 1 *KRAS*-negative patient (20%) experienced progression of disease, as opposed to 11 patients (57%) in the group with detectable mutant *KRAS* at baseline. Patients with stable or declining ctDNA levels after one cycle of chemotherapy had a significantly longer progression-free survival (7.2 vs. 2.6 months, $p = 0.037$) than patients with ctDNA on the rise.

In conclusion, our findings support the hypothesis according to which ctDNA has the potential to become a new biomarker for the monitoring of treatment efficacy in advanced pancreatic cancer. However, further research is required, as the various steps of the procedure must be standardised and this methodology needs to be validated in large clinical trials.

Chapter 1. Pancreatic cancer

1.1. Epidemiology

"If cancer is the emperor of all maladies, then pancreatic adenocarcinoma is the ruthless dictator of all cancers".[1]

Pancreatic ductal adenocarcinoma (PDAC), which represents more than 85% of tumours arising from the pancreas, is the most lethal common malignancy[2], with the lowest 5-year relative survival rate among the so-called "deadliest", or "recalcitrant", cancers – the subtypes which still fall under 50% survival rate nowadays[3]. Out of 100 patients diagnosed with PDAC, only 6 are still alive 5 years after diagnosis[4], compared to the current average of 69 for all types of cancer.

The most up-to-date data[5] indicate that pancreatic cancer is the eleventh most common malignancy newly diagnosed in males and the ninth in females. Despite its relatively low incidence, ranging from 1 to 10 cases for 100,000 people[2], it is the fourth leading cause of cancer-related deaths in both sexes at the present time (Fig. 1). The incidence of this neoplasm shows an upward trend, with a +0.8%/year rate for males and +2.0%/year for females respectively[5]. As a result, total deaths due to pancreas cancer have been projected to increase dramatically in the next years, resulting in pancreatic and colorectal cancers causing an equivalent number of deaths in 2020 and pancreatic adenocarcinoma becoming the second top cancer killer by 2030 (Fig.2)[6]. The estimated lifetime risk of developing pancreatic cancer is 1.47%; this risk increases with age, with onset occurring in the seventh and eighth decades most commonly[4].

1.2. Risk factors

The aetiology of pancreatic cancer is complex and multifactorial; a number of factors are known to increase the risk, some of which are modifiable and some non-modifiable.

The first category includes cigarette smoking, obesity, high animal fat intake, occupational exposure to nickel and chlorinated hydrocarbons, partial gastrectomy and recent-onset diabetes mellitus; age, race, sex, positive family history, chronic pancreatitis, and non-O blood group belong to the latter[4].

Cigarette smoking and family history are dominant among risk factors[7]. About 20% of PDACs are caused by cigarette smoking: current smokers have a 2.2-fold increased risk of pancreatic cancer compared to non-smokers, and the risk increases with the duration of the smoking habit and the number of cigarettes smoked[8].

It is estimated that 5 to 10% of pancreatic cancers have an inherited component. In the majority of cases, the genetic basis for familial aggregation has not been identified, but there are some familial syndromes which definitely confer an increased risk for the development of a pancreatic neoplasm[9]. For example, patients with hereditary pancreatitis caused by a mutation in the *PRSS1* or *SPINK1* genes, coding for trypsinogen and pancreatic secretory trypsin inhibitor respectively, have a 50-fold increased risk of developing pancreatic cancer[10].

Furthermore, there are many cases in which the syndrome is caused by a mutation in a tumour suppressor gene, such as *CDKN2A*, coding for the cyclin-dependent kinase inhibitor p16 (Familial Atypical Multiple Mole Melanoma syndrome), *MLH1*, *MSH2*, *MSH6*, involved in the DNA mismatch repair (Hereditary Non-Polyposis Colorectal Cancer syndrome), *BRCA2*, which plays a role in DNA double-strand breaks repair (Familiar Breast Cancer 2), *ATM*, a protein kinase activated by DNA double-strand breaks and recruiting tumour suppressor proteins such as p53 (Ataxia Telangiectasia), and *STK11* (Peutz-Jeghers syndrome)[9]. Germline *BRCA2* mutations account for the highest proportion of known causes of inherited pancreatic cancer, having been

identified in 5-17% of families with familial pancreatic cancer[11].

1.3. Pathogenesis

Pancreatic cancer arises as the result of a progression cascade involving multiple mutations. As in the polyp-to-adenocarcinoma sequence established in colon cancer, there is a progression from normal ductal epithelium, to duct lesions, to invasive ductal adenocarcinoma in pancreatic lesions (Fig. 3)[12]. Three different noninvasive lesions have been identified as precursors to PDAC: pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN), mucinous cystic neoplasm (MCN)[4,7].

PanIN is the most frequent noninvasive precursor to invasive PDAC. PanIN formation is preceded by acinar-to-ductal metaplasia[13]. A stepwise progression from low-grade to high-grade dysplasia is then recognisable moving from PanIN-1A and 1B onto PanIN-2 and 3: the increase in cytological and architectural atypia corresponds to incremental genetic alterations, which are the same found in invasive pancreatic cancer[13,14]. More than 90% of cases of PanIN of all grades have mutations in the *KRAS* oncogene: activating point mutations in *KRAS* codon 12, most often G12D, together with the overexpression of *Her-2/neu*, occur early in pancreatic duct lesions with minimal cytological and architectural atypia (PanIN-1A and 1B). Inactivation of the *CDKN2A* gene, coding for p16, is a feature of the moderate dysplasia stage (PanIN-2), while the inactivation of other tumour suppressors such as *p53*, *SMAD4*, and *BRCA2* characterises later stages (PanIN-3, high-grade dysplasia/carcinoma in situ)[12,15]. *KRAS* is mutated in more than 90% of PDACs; *CDKN2A* is the most frequently altered tumour suppressor gene, with loss of function in approximately 90% of tumours; *p53* is inactivated in a smaller proportion of malignancies, around 60-70%, and *SMAD4*, which mediates signaling downstream of the transforming growth factor β (TGFβ) receptor, is mutated in about 50% of pancreatic cancers. Apart from mutations in single genes, pancreatic cancer is characterised by abnormalities at a chromosomal level, such as

amplifications, deletions, rearrangements and telomere shortening; the latter is already present in the early stages of PanIN and is thought to contribute to tumour progression by enhancing chromosomal instability[16]. PanIN-1 lesions become very common with increasing age, while PanIN-3 is usually associated with invasive cancer[7].

PanIN lesions are too little (diameter < 5 mm) to be radiologically detected; in contrast, intraductal papillary mucinous neoplasms (IPMNs) are larger cystic tumours (diameter ≥ 5mm) which are more and more often being diagnosed because of the advances in pancreatic imaging[17,18]. Just as Pan-INs, non-invasive IPMNs are classified as either low-grade, intermediate-grade, or high-grade dysplasia (carcinoma in situ). Although IPMNs are only responsible for less than 15% of invasive PDACs, they offer the rare chance to identify a premalignant pancreatic lesion[4] and either resect it immediately or follow it up, still being able to cure any early invasive malignancy that may develop. Neoplasms arising from the main pancreatic duct (MD-IPMN) and from branch ducts (BD-IPMN) have a different risk of evolving into invasive PDAC: 40-92% for MD-IPMN, which should always be resected if the patient is deemed fit for surgery, compared to 15-25% for BD-IPMN. The latter may qualify for upfront resection or be managed via surveillance by periodic pancreatic imaging after radiological and histological evaluation depending on the risk stratification category they belong to (high risk, worrisome risk, low risk) according to the latest guidelines[19]. Like PanINs, IPMNs frequently harbour *KRAS* mutations (40-65%); in contrast, IPMNs hardly ever inactivate *SMAD4*. Moreover, 40 to 80% have activating mutations in the *GNAS* gene, coding for the G-protein subunit α_s, which activates adenylate cyclase to produce cyclic AMP[2,7].

Unlike PanIN and IPMN, mucinous cystic neoplasms (MCNs) do not communicate with pancreatic ducts. This kind of lesion is a less common precursor to pancreatic cancer made of mucin-producing epithelial cells and an associated ovarian-type stroma, is typical of women and has an associated invasive carcinoma in about one-third of cases[4].

As the most common oncogenic mutation in both premalignant precursors and PDAC (present in more than 90% of invasive tumours), *KRAS* activation has been the object of in-depth

investigation. The *KRAS* gene encodes a GTPase of the Ras family which, if activated, can recruit and activate proteins in the signal transduction pathways originating from growth factors' and other ligands' receptors, such as c-Raf and PI 3-kinase[20]. *KRAS* is a major driver of pancreatic tumorigenesis, contributing to cancer initiation by inhibiting the entrance of cells into a state of permanent growth arrest[21] and enhancing cell growth and reproduction (Fig. 4), while cancer progression requires the inactivation of tumour suppressor genes, which occurs at later stages. A high level of sustained activity of the oncogenic form of *KRAS* (e.g. $KRAS^{G12D}$) is necessary for earlier lesions to develop into pancreatic cancer. In a genetically engineered mouse model in which the mutant *KRAS* allele may be switched off at will, stopping *KRAS* activity causes massive cell death and proliferation arrest, with rapid tumour regression.

Moreover, *KRAS* regulates factors which play a role in the relationship between the malignant cells and the stroma, such as sonic hedgehog, interleukin-6 (IL-6) and prostaglandin E. Pancreatic ductal adenocarcinoma is associated with a desmoplastic stromal reaction: intratumoral connective tissue surrounding the cancer cells increases considerably, preventing chemotherapeutic drugs from penetrating into the cancer tissue. Sonic hedgehog signalling regulates the desmoplastic reaction of the stroma. In particular, pancreatic cancer cells closely interact with activated pancreatic stellate cells (PaSCs) for the latter to produce extracellular matrix and cause stromal fibrosis. Conversely, PaSCs enhance cancer cells proliferation and inhibit apoptosis and are believed to be at least partially responsible for the resistance of this malignancy to chemotherapy and radiotherapy[22–24]. This dense fibrosis is a hypovascular and hypoxic microenvironment the tumoural cell adapts to by shifting from aerobic metabolism to anaerobic glycolysis, a process which is in turn regulated by *KRAS*. Macropinocytosis and autophagy are enhanced, too, and this is another mechanism through which this oncogene guarantees the supply of metabolites to malignant cells (Fig. 4)[20,25].

Inflammatory markers such as IL-6 are increased in PDAC patients. The main sources of this cytokine are tumour-associated macrophages. Even though only a small minority of individuals diagnosed with pancreatic cancer have clinical evidence of chronic pancreatitis, there is histological

evidence of inflammation in tissue specimens, thus suggesting a possible injury-inflammation-cancer pathway[26]. The inflammatory stimulus activates PaSCs, which in turn recruit inflammatory cells such as neutrophils, macrophages and T cells, which produce IL-6; a possible effect of the inflammatory microenvironment on cancer progression is that IL-6 activates STAT-3 to promote the development of PanIN and PDAC.

1.4. Clinical presentation

Molecular genetics studies have shown that cancer progression in PDAC takes at least a decade from the initiating mutation to invasive carcinoma. At least five additional years are necessary for a clone with metastatic potential to emerge within the primary tumour[27], thus providing an extended time window in which the tumour could be identified before spreading to other organs.

However, one of the reasons why the prognosis of pancreatic cancer is so dismal, alongside its chemo- and radioresistance, is that diagnosis usually occurs at a late stage. Patients usually remain asymptomatic or have subtle and non-specific symptoms until the tumour invades surrounding structures[28]. The most common symptoms related to pancreatic cancer are asthenia (86% of patients), anorexia (83%), unexplained weight loss (85%), diffuse abdominal pain (79%), epigastric pain (71%), mid-back pain (49%), choluria (59%), jaundice (51%) (Table 1). Physical examination can reveal, apart from the above-mentioned jaundice, hepatomegaly (39%), a mass in the abdominal right upper quadrant (15%), ascites (5%), positive Courvoisier's sign (palpable distended gallbladder at the right costal margin; 13%) and Trousseau's sign of malignancy (recurrent migratory thrombophlebitis; 3%) (Table 1)[29].

The location of the mass within the pancreas influences how and how rapidly the tumour will manifest itself (Fig. 5). Neoplasms arising from the head, neck or uncinate process, which account for more than 60% of pancreatic malignancies, usually have an earlier presentation as a consequence of compression or infiltration of the common bile duct and consequent sudden-

onset obstructive jaundice[28]. The other two complaints that form the classic triad of signs and symptoms derived from a malignancy in the head of the pancreas are weight loss and abdominal pain. Weight loss can be due to various motives such as anorexia, maldigestion from pancreatic duct obstruction, and cachexia. Pain is usually referred to the epigastrium or right hypochondrium, with radiation to the sides and/or back and worsening after food intake[29]. Cephalic tumours can infiltrate the duodenum, causing gastric outlet obstruction or delayed stomach emptying, with associated nausea and early satiety.

In one-fifth to one-quarter of cases the lesion is located in the body or tail of the pancreas, left of the superior mesenteric vessels, and its primary manifestations are weight loss and pain. The latter arises when the tumour has reached considerable dimensions or has spread beyond the pancreas, for example to the peri-neuronal tissue or ganglia themselves. The pain has an insidious onset, with a gnawing visceral quality, is usually referred to the back and, with regard to its intensity, is severe. The duration of symptoms is usually of several months before diagnosis, compared to days or weeks for tumours of the head[4].

Up to 10% of patients with PDAC present with new-onset diabetes or worsening of pre-existing diabetes: the relationship between diabetes mellitus and pancreatic cancer is complex and controversial. In several studies, the prevalence of diabetes in PDAC has been reported to be between 40 and 75%[30–32]. Diabetic subjects have been found to have a 30% higher risk of pancreatic cancer persisting for more than 20 years after the initial diagnosis of diabetes, suggesting that diabetes is not merely a marker of pancreatic dysfunction as a result of neoplasia[33,34]. The cause of the diabetogenic state is still not well understood, but diabetes is sometimes cured by resection of the tumour, thus suggesting that it is indeed caused by the neoplasm itself[35]. This kind of diabetes would be classified as type 3c, pancreatogenous diabetes, as opposed to the more common type 2 late-onset diabetes. Patients aged over 50 with new-onset diabetes have an 8-fold higher risk of developing pancreatic cancer within 3 years of the diagnosis compared to the general population[35,36]: as a result, any of the above-mentioned symptoms in the presence of late-onset

diabetes should strongly alert the clinician to the possibility of a malignancy arising from the pancreas. However, most patients with newly diagnosed late-onset diabetes do not have pancreatic cancer and, apart from weight loss, few clinical clues exist to suspect this deadly underlying condition in a patient who has recently received a diabetes diagnosis[31,35].

With regard to the differential diagnosis of pancreatic cancer, choledocholithiasis, pancreatic pseudocysts, chronic pancreatitis, biliary tract and ampullary carcinomas, and other pancreatic malignancies such as pancreatic lymphoma must be ruled out[28].

PDAC is usually diagnosed at a late stage; diagnoses posed at an early stage are rare and usually occur for small tumours located in the head near the common bile duct which is thereby occluded, causing jaundice, or are incidental, following abdominal imaging performed for other reasons.

1.5. Diagnosis

Diagnosis and staging of pancreatic cancer come together with the necessity to evaluate the possibility of surgical resection, this being the only chance of cure.

Diagnostic certainty is gained only through histological examination of a tissue sample. In the case of non-resectable advanced disease, histopathological diagnosis must be obtained before starting medical treatment. In case clinical and radiological features make highly likely the presence of a resectable malignancy, pre-surgery histological diagnosis is not mandatory. Endoscopic ultrasonography-guided fine-needle aspiration (EUS-FNA) biopsy has a high diagnostic accuracy (85-90%) for pancreatic cancer[33]. Alternatively, a tissue specimen can be obtained via CT- or US-guided percutaneous biopsy.

A number of different imaging techniques have been evaluated to pose a diagnosis and stage the disease (Fig. 6)[37], while also playing a role in monitoring the response to chemotherapy:

- Abdominal ultrasound (AUS) is usually the first-line imaging technique used when

approaching a patient with clinical suspicion of pancreatic cancer, especially when jaundice is the main sign at presentation. However, the diagnostic accuracy of ultrasonography greatly depends on the operator's experience and the patient's condition in terms of obesity and bowel gas, thus limiting its adoption for staging and assessing resectability. The sensitivity and specificity of transabdominal ultrasound for pancreatic cancer range from 75% to 89% and from 90% to 99% respectively[38]. The involvement of the major vessels can be assessed coupling standard AUS with a Doppler ultrasound study[39].

- Multidetector-row computed tomography (MDCT) with contrast medium is at the present time the imaging technique routinely used for the radiological diagnosis of suspicious pancreatic lesions (e.g. an hypoechoic lesion found on AUS previously performed), assessment of vascular invasion and resectability, and diagnosis of metastases[40]. Pre-contrast scans can show calcifications in the pancreas, thus playing a role in differential diagnosis by ruling in chronic pancreatitis. The vascular-poor and hypo-perfused pancreatic tumour enhances poorly compared to the surrounding parenchyma in the arterial phase, resulting in the lesion being visualised as a hypoattenuating area, and gradually enhances with delayed images. The arterial phase images also allow the assessment of arterial vessels involvement, while the venous phase images are used to identify peritoneal foci and liver metastases[37]. Hypoattenuation has a sensitivity of 75% and specificity of 84% in diagnosing pancreatic cancer; other helpful CT findings are pancreatic and common bile duct dilatation, ductal interruption, distal pancreatic atrophy, pancreatic contour abnormalities[41]. Overall, MDCT has an accuracy of roughly 90% for diagnosis and an accuracy of about 85% in determining resectability[42,43].

- Gadolinium-enhanced magnetic resonance imaging (MRI) has been shown to be non-inferior to MDCT, being their sensitivity (83-85% vs. 83%) and specificity (63% vs. 63-75%) similar[44]. MRI is particularly useful to assess peri-pancreatic infiltration, due to the high contrast resolution of this technique to differentiate pancreatic parenchyma and the

surrounding fat. Magnetic resonance cholangiopancreatography (MRCP) is frequently performed in conjunction with abdominal MRI, does not need intravenous contrast and allows non-invasive delineation of the pancreatic duct and biliary tract[45]. It is used for the diagnosis of small pancreatic masses and is gradually replacing the invasive endoscopic retrograde cholangiopancreatography (ERCP), although MRCP does not allow tissue sampling.

- ERCP is a sensitive tool to visualise the biliary tract and pancreatic duct, with sensitivity and specificity of 92% and 96% respectively for diagnosing pancreatic cancer. Tissue samples may be collected through forceps biopsy or brush cytology. Its diagnostic role has been decreasing lately because of the 5-10% risk of significant complications, such as acute pancreatitis and gastrointestinal or biliary perforation, and the availability of safer alternatives such as MRCP. ERCP is currently mainly used to insert a plastic or metal biliary stent for palliation of jaundice and pruritus due to tumour obstruction of the common bile duct.

- 18F-fluorodeoxyglucose positron emission tomography (^{18}FDG-PET) has several limitations in diagnosing pancreatic carcinoma, including possible false-negative results in hyperglycaemia and possible false-positive results in pancreatitis. Two meta-analyses showed that ^{18}FDG-PET plus CT has no major advantages over routine methods[46,47]. However, it can prove useful in other settings, i.e. confirming a complete remission or differentiate post-surgical scar tissue and local recurrence.

- Endoscopic ultrasonography (EUS) has been reported to be superior to MDCT in diagnosing pancreatic cancer, with a sensitivity of 98-100% compared to about 85% for MDCT[48,49]. This invasive procedure requires the introduction of an endoscope in the upper digestive tract; the endoscope is equipped with a high-frequency ultrasound transducer which allows high-resolution imaging of the pancreas and surrounding blood vessels. Tissue sampling for diagnostic cytology/histology can be undertaken at the time of the

endoscopic ultrasound (EUS-FNA). EUS is highly operator-dependent and requires expensive equipment; therefore, the use of this method is still not widespread.

A role in the diagnosis of PDAC is also played by serum tumour markers. The most useful and widely adopted is the cancer-associated antigen 19.9 (CA19.9), an epitope of blood group Sialis Lewis antigen. When used in patients with clinically- and radiologically-based suspicion for pancreatic cancer, CA 19.9 serum levels have a sensitivity and specificity for diagnosis ranging from 79% to 81% and from 82% to 90% respectively[50]. However, CA19.9 is not useful as a screening marker because of low positive predictive value, which is below 1%. The sensitivity is limited for small-sized tumours, while it is positive in about 80% of cases of advanced pancreatic cancer. CA19.9 is falsely increased in a number of pancreaticobiliary disorders, most commonly obstructive jaundice[50], given the fact that CA19.9 is partly excreted through bile, but also in acute and chronic pancreatitis and pancreatic pseudocyst. A preoperative CA19.9 value >100 U/mL implies likely locally advanced or metastatic disease, while a concentration of <100 U/mL suggests a resectable tumour[50]. Also, a preoperative CA19.9 level > 100 U/ml has been shown to be a predictor of early recurrence and poor prognosis after surgical resection[51]. A drop in postoperative CA19.9 serum levels by at least ≥20-50% from baseline following surgical resection or chemotherapy is associated with longer survival[50]. In patients with CA19.9 elevation, this marker may be used as a predictor of prognosis, overall survival, and in monitoring the response to surgical and medical treatment; as a result, it is advisable to measure CA19.9 serum levels before surgery, before the start of adjuvant chemotherapy and during follow-up[52].

1.6. Staging

Unlike many other malignancies, imaging is the primary means through which the stage of pancreatic cancer is determined[53]. Definite pathological staging is possible only after surgical resection, which, however, cannot be performed in about 80% of cases. As a result, pancreatic

cancer is often staged by clinical rather than formal TNM classification. Abdominal helical CT scans and chest X-rays (or chest-abdomen-pelvis CT) are the radiological bases of staging, given the fact that pancreatic cancer most often metastasises to the liver, abdomen, and lungs[2,4].

According to the American Joint Committee on Cancer Tumour-Node-Metastasis classification (Table 2), potentially resectable tumours are classified as T1-T3, whereas T4 tumours, which involve the superior mesenteric artery (SMA) or celiac axis, are unresectable[54]. Masses involving the superior mesenteric vein (SMV), portal vein (PV) or splenic vein are classified as T3 because veins are possibly susceptible of resection and reconstruction. Resectable tumours without lymph node metastases belong to stage I and IIA; resectable tumours which have metastasised to regional lymph nodes (N1) are classified as stage IIB, while stage III is composed of unresectable, locally advanced pancreatic cancers (LAPC). Metastatic disease (stage IV) includes both cases of radiological or histological evidence of metastasis to distant organs such as liver, lungs etc. and positive peritoneal washing cytology.

The other main staging system is the one which follows the guidelines released by the National Comprehensive Cancer Network: pancreatic cancers form a continuum from resectable to unresectable according to the involvement of nearby structures and the presence or absence of distant metastases (Fig. 7)[55]. The direct reference to surgical intervention is because margin-negative surgical resection is the only opportunity to cure pancreatic adenocarcinoma and therefore accurate determination of resectability is of momentous importance for optimal management. Locoregional disease can be divided into three categories: resectable, borderline resectable, and locally advanced (unresectable), according to the extent of local invasion. Patients with borderline resectable disease initially may not be operable, but, with some cytoreduction (e.g. neoadjuvant chemotherapy or chemoradiotherapy) may achieve a margin-negative resection[55].

Cases in which there is no evidence of distant metastases and tumour extension to the SMV and PV and there are clear fat planes around the celiac axis, the hepatic artery, and SMA should be categorised as having localised resectable disease (Table 4). Radiographic findings definitely

contraindicating surgery include distant metastases, major venous thrombosis of the PV or SMV extending for several centimetres, and circumferential encasement of the SMA, celiac axis or proximal hepatic artery[56].

There is no general consensus on the definition of borderline resectable pancreatic cancer (BRPC). In 2014, the NCCN Pancreatic Adenocarcinoma Panel declared that "no perfect definition of borderline resectable disease is currently possible because of insufficient data"[55]. However, according to the latest NCCN criteria, BRPC is defined by radiological findings of venous involvement of the SMV or PV with vein distortion or narrowing, or occlusion of the vein with suitable proximal and distal vessel allowing for safe resection and replacement[55]. In fact, the involvement of a short segment of PV or SMV permits successful resections thanks to venous reconstruction[57], while longer segment involvement or occlusion at the SMV-PV confluence make resection technically prohibitive. As for arterial involvement, BRPC can be characterised by radiological evidence of encasement of a short segment of the hepatic artery, without evidence of tumour extension to the celiac trunk, and/or tumour abutment of the SMA involving 180° or less of the artery circumference (Table 4)[55]. Other authors define BRPC as (1) tumour abutment (≤180°) of the SMA or celiac artery, (2) encasement (>180°) of the SMV and/or PV with an option for venous resection/reconstruction, (3) presence of indeterminate liver/lung lesions, regional lymph node metastases, or a patient with a performance status deemed ineligible for immediate surgery[58].

Only 10 to 20% of patients can be classified as resectable (stage I-II) at diagnosis[59], while approximately 5-7% as borderline resectable[60]. Among those who undergo surgical resection and have tumour-free R0 margins, the 5-year survival rate is still very low, ranging between 10 and 25%, with a median survival of 20-23 months[59].

One-quarter to one-third of cases fall within the locally advanced tumour category (stage III), which is associated with a median survival of 8 to 14 months, with most of the patients progressing to metastatic disease within the first year.

The remaining 45-55% of subjects present with metastatic disease (stage IV), for which the prognosis is still extremely dismal, with a median life expectancy inferior to one year from diagnosis[4,7] (Table 3).

1.7. Management

Patients with pancreatic cancer need to be managed by a multidisciplinary team including medical and radiation oncologists, surgeons, radiologists, pathologists, pain management specialists, dieticians and, in the terminal stages, palliative care experts[61].

1.7.1. Surgical management of resectable tumours

When the tumour is resectable, surgery remains the treatment of choice, although pancreatic cancer is a systemic disease at the time of diagnosis and there is preliminary evidence that patients may so benefit from neoadjuvant or peri-operative medical treatment[62,63]. Surgery is presently the only potentially curative intervention and can result in significantly longer survival compared with other treatment options[33]. Patients who undergo surgery with negative margins and subsequently receive adjuvant therapy have the best chance of cure. However, outcomes are still far from optimal: even after multimodality treatment including surgical resection, 5-year survival rates only reach 25 to 30% at best[64].

The primary goal of surgery is to obtain the total removal of the tumour, identified by resection margins negative for cancer cells. The Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC) classify possible pathological findings after surgery as negative resection margins (R0), microscopic tumour infiltration (R1) and macroscopic residual tumour (R2). American guidelines define microscopic residual tumour as the presence of cancer cells at the surface of the resection margin (0 mm rule), whereas the British Royal College of Pathology (RCPath) define R1 as the presence of malignant cells within 1 mm of the resection

margin (1 mm rule)[65]. The International Study Group of Pancreatic Surgery (ISGPS) has recently recommended adhering to the latter[33], as there is evidence that a minimum clearance of more than 1 mm is required for effective disease control, as survival after resection with less than 1-mm margin (R0-close resection) is worse than that of resection with more than 1-mm margin (R0-wide resection) and similar to survival after R1 resection[66].

The assessment of resectability by radiological evaluation of the extent of local invasion and, in particular, vessel involvement, is of critical importance to select the patients with a high probability of achieving R0 resection, who are the ones eligible for immediate surgery; nonetheless, R1 resection is a postoperative finding in about 30% of patients. Subjects with BRPC have a high likelihood of R1 resection and, as a consequence, should not be regarded as good candidates for upfront surgery[67].

The location of the malignancy influences the type of surgery which will be performed. Surgical techniques for pancreatic cancer include pancreatoduodenectomy (Whipple procedure) for tumours located in the uncinate process or head of the pancreas, distal pancreatectomy with splenectomy for lesions in the body or tail of the pancreas, and total pancreatectomy for those tumours which diffusely involve the gland[28]. Laparoscopy has been shown to reduce the morbidity associated with distal pancreatectomy without negative oncologic outcomes[68,69] and is, therefore, a viable approach for selected lesions in the tail of the pancreas; however, there is still insufficient evidence to recommend it and conventional open surgery remains the standard of care at the present time[67]. Frequent postoperative complications are pancreatic anastomosis leaks and delayed gastric emptying: research has been conducted on which operative approaches (e.g. pylorus-preserving, subtotal stomach-preserving) have the least likelihood of complications after pancreatoduodenectomy, but such studies have found no clear advantage of one technique over another[7].

The hospital volume has been found to be associated with risk of complications, mortality, length of hospital stay, margin status, and survival[70,71]: as a result, surgery for pancreatic cancer

should be carried out in dedicated centres performing an adequate number (>15-20) of pancreatic resections per year[67].

Resection of the SMV or PV to achieve macroscopic tumour clearance can be performed safely with acceptable operative morbidity and mortality[72,73]. Instead, arterial resections are associated with increased morbidity and mortality and are not recommended[67], although some surgeons with great expertise in the field of vascular reconstruction consider them feasible.

Lymph node involvement represents a major prognostic factor. Therefore, standard lymphadenectomy must be performed during surgery ensuring that an appropriate number (at least 12-15) of regional lymph nodes are collected. The total number of lymph nodes analysed, the number of metastatic lymph nodes, and the ratio between them (lymph node ratio) should be reported, as this is one of the most significant prognostic parameters[74,75].

In fact, other factors suggesting a poor prognosis with early recurrence and mortality include high tumour grade (G3-G4), a large size tumour, high (>200 U/ml) preoperative CA19.9 levels, persistently elevated postoperative CA19.9 levels, R1 or R2 resection[76]. In contrast, outcomes are more favourable if the tumour is relatively small (<3 cm), well differentiated (G1-G2) and has not metastatised to lymph nodes (N0)[28].

Nevertheless, it should be noted that, even under optimal conditions, the median survival of patients who undergo surgical resection for PDAC only is less than 20 months and the 5-year survival rate is about 10%[61], thus advocating for the need of subsequent additional treatment. This is because, unfortunately, development of distant metastases, most frequently to the liver, and local recurrence are extremely common.

1.7.2. Adjuvant treatment for patients with surgically resected pancreatic cancer

A number of studies have evaluated which chemotherapy and chemoradiation (CRT) regimens could be deemed appropriate for adjuvant treatment.

In the European Study group for Pancreatic Cancer 1 (ESPAC-1) trial, a two-by-two factorial design study, patients who underwent macroscopically radical resection (R0 or R1) were randomised in four treatment arms: chemotherapy (5-fluorouracil and folinic acid), CRT (20 Gy over a two-week period plus 5-fluorouracil), both, or observation only[59]. The results indicated that patients receiving adjuvant chemotherapy had a better 5-year survival rate (21% vs. 8%) and median survival (20.1 vs. 15.5 months), thus demonstrating a clear survival benefit for chemotherapy, irrespective of whether the patient had received CRT or not, and reinforcing the hypothesis that, being pancreatic cancer a systemic disease at the time of diagnosis, it needs an effective systemic treatment. Worrisomely, patients treated with CRT, alone or associated with systemic chemotherapy, had a worse outcome, the 5-year survival rate being 10% for patients who received CRT and 20% for those who did not and the median survival respectively 15.9 and 17.9 months ($p=0.05$). The authors claimed that CRT delayed the start of systemic chemotherapy, thus reducing the benefit deriving from its administration as soon as possible after surgery; these data, albeit criticised for study design issues, have brought to a fall in the use of adjuvant chemoradiation in Europe.

The Charité Onkologie 001 (CONKO-001) study was a randomised controlled trial whose purpose was to compare adjuvant gemcitabine with no postoperative medical treatment after curative-intent resection of pancreatic cancer[77]. Gemcitabine had been introduced as the standard first-line treatment in metastatic PDAC in the late 1990s, with both a significant rise in survival and an excellent safety profile, with a low incidence of grade 3 and 4 toxicities[78]. In the CONKO-001 trial, gemcitabine was administered for 6 cycles, every cycle consisting of 3 weekly intravenous infusions of gemcitabine 1000mg/m^2 followed by a 1-week pause. The results of the qualified analysis, from which patients with even minor protocol violations were excluded, demonstrated improved disease-free survival (DFS) and overall survival (OS) in the gemcitabine arm compared to the observation-only arm: 13.7 vs. 6.9 months ($p<0.001$) and 24.2 vs. 20.5 months ($p=0.02$) respectively. At 5 years, approximately twice as many patients in the gemcitabine group compared

to the control group were still alive (22.5 vs. 11.5%). Adjuvant gemcitabine was found to have a beneficial effect on DFS both in patients with R0 (13.1 vs. 7.3 months) and R1 (15.8 vs. 5.5 months) resections. Following this study, adjuvant chemotherapy with gemcitabine was established for the postoperative treatment of PDAC.

The ESPAC-3 trial compared the two chemotherapeutic agents which had been previously examined in the adjuvant treatment of pancreatic cancer: 5-fluorouracil (5-FU) and gemcitabine. This was the largest adjuvant trial ever conducted, in which 1088 patients were randomised to receive either 6 cycles of 5-FU and folinic acid (420 mg/m^2 + 20 mg/m^2 for 5 consecutive days every 28 days) or gemcitabine (1000 mg/m^2 once weekly for 3 out of 4 weeks)[79]. No significant difference in DFS (14.1 vs. 14.3 months, non-significant) and OS (23.0 vs. 23.6 months, non-significant) were reported, but treatment-related grade 3 and 4 toxicities were more frequent in the 5-FU plus folinic acid arm (14% vs. 7.5%). Therefore, both the American Society of Clinical Oncology (ASCO) and the European Society for Medical Oncology (ESMO) recommend either 5-FU plus folinic acid or gemcitabine for adjuvant therapy, although the latter is usually preferred because less toxic[61,67]. It is worth noting that, as opposed to early initiation, completion of all six cycles of adjuvant chemotherapy, was reported to be a positive prognostic factor. The outcome did not differ if the start of chemotherapy was delayed up to 12 weeks after surgery; as a result, the start of adjuvant treatment may be deferred until the patient has fully recovered from the major stress of pancreatic surgery[80].

The Japanese Adjuvant Study group of Pancreatic Cancer 01 (JASPAC-01) trial compared gemcitabine and the oral fluoropyrimidine S-1, which was developed in Japan and contains a 5-FU prodrug, tegafur[81]. The 5-year survival rate was 24.4% in the gemcitabine arm and, intriguingly, 44.1% in the S-1 group. Since 5-FU metabolism is different in Asian and Caucasian populations, further research is needed to assess whether these results are valid in a non-Asian population.

The results of the ESPAC-4 trial have been recently presented at the ASCO 2016 Annual Meeting[82]. This trial was aimed to examine the combination chemotherapy with gemcitabine and

capecitabine (GEM/CAP), which had previously been found to improve the survival of a subgroup of patients with metastatic pancreatic adenocarcinoma with a good performance status (Karnofsky score of 90 to 100) compared to gemcitabine only[83], in the adjuvant setting. Patients with pancreatic cancer were randomised within 12 weeks of surgery to have either six 4-week cycles of intravenous gemcitabine alone or gemcitabine plus oral capecitabine. The study had positive results, with a median survival for patients treated with gemcitabine plus capecitabine of 28.0 months compared to 25.5 for gemcitabine only and a 5-year survival rate of 28.8% vs. 16.3%[82]. The results of this trial are promising; in the future, this combination may be adopted for the adjuvant treatment of the subgroup of patients fit for polychemotherapy.

By way of a conclusion, at the present time, it is recommended that all patients who underwent surgery for PDAC and did not receive neoadjuvant treatment receive adjuvant chemotherapy with 6 cycles of either gemcitabine or 5-FU plus folinic acid, if not contraindicated for medical or surgical reasons[61,67]. The use of adjuvant chemoradiation is controversial; since its primary benefit is the supposed decreased risk of local recurrence, this treatment modality may be considered for patients with R1 resection and/or positive lymph nodes after 4-6 cycles of systemic adjuvant chemotherapy[61].

1.7.3. The role of neoadjuvant therapy in borderline resectable pancreatic cancer

In patients with BRPC, resection may be technically feasible but with a high likelihood of microscopically positive margins (R1 resection), this being a major negative prognostic factor for overall survival[84].

Neoadjuvant (preoperative) therapy has been investigated in depth as a strategy to obtain the downsizing and downstaging of the mass, thus possibly allowing successful R0 resection, and better selection of candidates, avoiding surgery which would very unlikely provide any clinical benefit for patients with particularly biologically aggressive tumours who rapidly develop distant metastases[85].

Neoadjuvant therapy is recommended by ASCO and ESMO for the management of BRPC; however, there is no consensus on which should be the standard of care because of the lack of randomised phase III studies[61,67]. Three meta-analyses based on phase II studies have been performed up to the present day. In their work published in 2010, including 111 trials and 4394 patients with primarily resectable pancreatic cancer, BRPC and LAPC, Gillen and colleagues[85] reported an overall resection rate of 33.2% for tumours not deemed primary resectable (BRPC/LAPC) after neoadjuvant chemotherapy or CRT (no separate analysis for BRPC and LAPC was performed). Neoadjuvant chemotherapy was administered in 96.4% of the studies considered (the most frequently adopted agents were gemcitabine, 5-FU, and platinum derivatives), whereas neoadjuvant radiotherapy was applied in 93.7% of the trials, with doses ranging from 24 to 63 Gy. The median OS for patient with BRPC/LAPC who underwent resection after neoadjuvant treatment was 20.5 months, not significantly shorter than that of primarily resectable patients treated with upfront surgery and adjuvant therapy (23.3 months). A second meta-analysis published in 2011 by Assifi et al.[86] analysed a much smaller number of studies: 14 phase II clinical trials. Eight studies used gemcitabine-based chemotherapy regimens, while the remaining 6 trials adopted 5-FU-based regimens; patients received neoadjuvant radiotherapy in 85% of the studies, with doses ranging between 30 and 50.4 Gy. The authors reported similar findings to those of Gillen and collaborators: following neoadjuvant treatment, resection rate was 31.6% for BRPC/LAPC patients, and the median survival in resected patients was 22.3 months. Altogether, these two studies strongly suggest that patients who are not ideal candidates for upfront surgery because of the local extent of the disease (BRPC and even LAPC) should be offered neoadjuvant therapy and then re-evaluated for resection[85,86]. Sequential treatment with induction chemotherapy aiming to improve the systemic control of the disease and subsequent chemoradiation to sterilise surgical margins and reduce the rate of R1 resections might be used[87]. At the present time, the only recommended CRT treatment is the combination of radiotherapy and capecitabine, although also 5-FU, gemcitabine or gemcitabine plus oxaliplatin have been used.

With regard to establishing which chemotherapy regimen should be offered for neoadjuvant treatment in patients with BRPC, the above-cited works are already outdated because they were carried out before two new regimens, FOLFIRINOX (a combination of 5-FU, folinic acid, irinotecan, and oxaliplatin) and gemcitabine plus nab-paclitaxel, were found to have significant activity in advanced pancreatic cancer (see *1.7.4, Treatment of metastatic pancreatic cancer*). These regimens, therefore, offer great promise as regards their adoption in patients with localised disease who might thus be able to undergo radical surgery and have a significantly longer survival. The most recent meta-analysis[88], conducted by an Italian group, examined 13 trials regarding the use of neoadjuvant FOLFIRINOX in BRPC and LAPC: a total of 253 patients received FOLFIRINOX with or without radiotherapy before being re-evaluated for surgery. The rate of resection for BRPC patients was 68.5%, and the percentage of R0 resections was 69.5%. The authors, therefore, proposed that FOLFIRINOX could become a new standard-of-care in patients with BRPC fit for polychemotherapy.

However, in the absence of conclusive evidence from randomised clinical trials, current recommendations are to include patients with BRPC in clinical trials whenever possible. Otherwise, a sequence of neoadjuvant chemotherapy (possible regimens: gemcitabine or FOLFIRINOX) and chemoradiation prior to re-evaluation for radical surgery seems to be the most appropriate way to manage borderline resectable lesions at the moment[61,67].

1.7.4. Management of locally advanced pancreatic cancer

When patients are not eligible for upfront resection but do not have overt metastatic disease, a multidisciplinary team should evaluate whether the tumour can be considered as BRPC or as truly unresectable, thus falling under the category of locally advanced pancreatic cancer (LAPC). The average OS for patients with LAPC at presentation has been reported to be less than 1 year in a number of studies. However, patients treated with chemotherapy alone in the more recent LAP07 trial, which only recruited patients with LAPC, had a median OS of 16 months[89]. This difference

may be due to more active regimens used to treat tumours which progress to develop distant metastases.

There is still a high degree of controversy on how these patients should be managed. According to current ESMO guidelines, 6 cycles of gemcitabine are still to be considered the standard of care for patients with LAPC. The first cancer-directed treatment should be systemic chemotherapy; patients with disease control after 3–6 months of chemotherapy could be treated with consolidative chemoradiotherapy[67].

These recommendations come as the result of decades of trials comparing upfront CRT with chemotherapy alone and induction chemotherapy (ICT) followed by CRT.

In one of the most recent studies, the FFCD/SFRO trial, patients were randomised to receive either gemcitabine monotherapy or a nowadays outdated CRT regimen (60 Gy plus infusional 5-FU and cisplatin) followed by maintenance gemcitabine. The outcomes concerning terms of overall survival (8.6 months vs. 13 months, $p = 0.03$) and severe toxicities were worse in the CRT arm, possibly because of the unfavourable safety profile associated with such a high dose of radiation, exceeding the tolerance of the peri-pancreatic organs[90].

The ECOG E4201 randomised phase III trial was aimed to compare gemcitabine alone with gemcitabine-based CRT followed by maintenance gemcitabine, but it had to close due to low accrual rate after the inclusion of 74 patients[91]. In contradiction with the results of the FFCD-SFRO trial, the analysis of the patients included in the ECOG E4201 trial showed that median OS was better in the CRT arm (11.1 months vs. 9.2 months, $p = 0.04$). However, again, grade 4 toxicities were more frequent in the CRT arm. The ECOG E4201 trial would point out a superiority of gemcitabine to 5-FU as a radiosensitizer in LAPC, while a randomised phase II study indicated that capecitabine could be less toxic and more active than gemcitabine in this setting[92].

The reason why ICT is recommended to be given before chemoradiation is that the low doses of chemotherapeutic agents used as radiosensitisers in CRT fail to control microscopic systemic disease efficiently, so approximately one-third of patients with LAPC develop distant metastases

within 3 months. Patients who do not progress after 3-4 months of systemic chemotherapy may then potentially benefit from CRT, as shown by two large retrospective studies[93,94]. The international phase III trial LAP07 compared gemcitabine with gemcitabine plus erlotinib as induction and maintenance chemotherapy (no significant difference in OS) and was also intended to look into the role of CRT in patients with no progression after ICT, as opposed to the continuation of chemotherapy for two more cycles. The results indicated that the OS of patients treated with CRT after 4 months of induction chemotherapy is not significantly different from that of patients who go on receiving systemic chemotherapy, even though there was a trend towards longer PFS in the CRT arm (median PFS 9.9 months vs. 8.4 months, p=0.06) and locoregional tumour progression was less common in the CRT group (32% vs. 46%, p = 0.035)[89]. In conclusion, these data highlight that standard CRT improves local control (LC) but not OS. However, LC is essential for patients with LAPC: up to 30% of patients with LAPC could die as a result of local progression. New radiation techniques, such as stereotactic body radiation therapy (SBRT), could prove instrumental in overcoming the limitations of CRT[95]. SBRT has been shown to offer excellent LC (1-year LC rate of 78%) with minimum acute and late grade ≥2 gastrointestinal toxicity rate in a recent multicenter phase II study[96], as a result of effective delivery to the tumour tissue and better sparing of the peri-pancreatic organs such as the duodenum. SBRT enables the delivery of ablative radiation doses (in the above-mentioned study, 33 Gy) in ≤5 sessions; patients with disease control after induction chemotherapy could receive SBRT and then quickly go back to fully-intensive systemic chemotherapy, avoiding the delay associated with conventionally fractioned CRT[95]. This treatment strategy is the object of a phase III trial underway at the present time (NCT01926197)[97].

Newer regimens such as FOLFIRINOX and gemcitabine plus nab-paclitaxel have not been examined in randomised controlled trials in the setting of LAPC yet. However, given that they already have been used quite extensively, and the studies in patients with metastatic PDAC have clearly demonstrated their efficacy and safety in that setting, according to the latest ASCO

guidelines, they may be recommended for people with LAPC with good performance status (ECOG PS 0 or 1), while gemcitabine only or gemcitabine plus capecitabine are a better option in patients with a borderline performance status[98]. In particular, the findings of a number of small retrospective and prospective studies suggest that FOLFIRINOX could obtain an impressive response rate in patients with LAPC, and may make a small but significant proportion of patients with LAPC resectable. In two meta-analyses regarding the use of FOLFIRINOX as induction chemotherapy in LAPC, just above one-quarter of patients underwent surgery after FOLFIRINOX (pooled resection rates: 26.1% and 25.9% respectively)[88,99]. In the latest, Suker and co-workers reported a median OS of 24.2 months for patients who received FOLFIRINOX[99], while several studies indicated a range of 6–13 months for patients with LAPC treated with gemcitabine. However, none of the studies considered was a randomised trial, so higher-level evidence is required. Findings from the ongoing phase III PRODIGE 29/NEOPAN trial (NCT02539537)[100], which compares FOLFIRINOX with gemcitabine in LAPC, may provide a definite insight into the efficacy of this regimen in locally advanced pancreatic cancer.

1.7.5. Treatment of metastatic pancreatic cancer

Chemotherapy is the mainstay of the treatment of metastatic pancreatic ductal adenocarcinoma (mPDAC). A pivotal study by Burris and co-workers[78] established gemcitabine as the standard of care for patients with mPDAC in 1997. 126 patients were randomised to receive either gemcitabine or bolus 5-FU. The primary endpoint of the study was clinical benefit response, resulting from the combination of measurements of pain (intensity, analgesic consumption), Karnofsky performance status, and weight. A significant difference was found as regards this parameter, with clinical benefit response achieved in 23.8% of patients treated with gemcitabine and only 4.8% of patients treated with 5-FU, thus demonstrating that gemcitabine is more effective than 5-FU in alleviating disease-related symptoms in mPDAC. An additional finding was an objective response rate of 5.4% for the gemcitabine arm, compared to 0% of the 5-FU arm; median survival was slightly but

significantly higher in the patients who received gemcitabine, at 5.65 months, as opposed to 4.41 months for the ones treated with 5-FU, the 1-year survival rates being 18% and 2% respectively. These results highlight a modest survival advantage with gemcitabine.

Gemcitabine is a nucleoside analogue similar to cytidine; it is usually administered weekly at a dose of 1000 mg/ m^2 for 3 every 4 weeks. Treatment is generally well tolerated; adverse reactions include dose-limiting myelosuppression (neutropenia, thrombocytopenia, although in some patients thrombocytosis can also occur), flu-like symptoms (fever, muscle pain, chills, and fatigue), mild nausea, vomiting, peripheral oedema, and skin rash[101].

The past few years have witnessed a breakthrough in the treatment of advanced pancreatic cancer. Two new chemotherapy regimens have been proven to be significantly more active than gemcitabine alone in large, well-executed, randomised phase III trials: FOLFIRINOX and gemcitabine plus nab-paclitaxel[102,103].

FOLFIRINOX, as mentioned above, is a combination of three chemotherapeutic agents, 5-FU, irinotecan, and oxaliplatin, plus folinic acid (also called leucovorin, LV), a derivative of tetrahydrofolic acid which enhances the action of 5-FU. Oxaliplatin is administered first (85 mg/m^2) as a 2-hour intravenous infusion, with the addition, after 30 minutes, of irinotecan at a dose of 180 mg/m^2, given as a 90-minute infusion, followed by an intravenous bolus of 5-FU (400 mg/m^2) and then continuous intravenous infusion of 2400 mg/m^2 of 5-FU over a 46-hour period thanks to the use of an elastomeric infusion pump and a central line. Overall, the administration of this chemotherapy regimens lasts 48 hours and is repeated every 2 weeks.

In the PRODIGE4/ACCORD11 study, a multicenter phase II/III trial carried out in France, whose outcomes were the subject of a paper published in 2011 by Conroy and colleagues[102], 342 patients with histologically confirmed mPDAC not previously treated with chemotherapy were randomised to receive either FOLFIRINOX or gemcitabine. Patients had to be fit for aggressive chemotherapy so as to receive FOLFIRINOX. As a results, in order to be eligible for inclusion, they had to be 75 years old or younger, have adequate bone marrow, liver and kidney function, have

bilirubin levels <1.5 times the upper limit of the normal range (<1.5 x ULN), and have an Eastern Cooperative Oncology Group performance status (ECOG PS) of 0 or 1. An ECOG PS 0 indicates that the patient is either fully active and able to carry on all pre-disease activities without restriction, while a patient with ECOG PS 1 is restricted in physically strenuous activity but is ambulatory and able to carry out work of light or sedentary nature[104]. Notably, in the population considered, more patients had a tumour located in the body or tail of the pancreas (59%) than in the head (38%), probably because bilirubin levels had to be normal or subnormal for inclusion.

FOLFIRINOX was found to be superior to gemcitabine in all outcome measures. The median OS was 11.1 months in the FOLFIRINOX group, as opposed to 6.8 months for the gemcitabine group (hazard ratio for death, 0.57; 95% CI, 0.45 to 0.73; p<0.001); median PFS was reported to be 6.4 months in the in the FOLFIRINOX group, compared to 3.3 months for the gemcitabine group (hazard ratio for disease progression, 0.47; 95% CI, 0.37 to 0.59; p<0.001) (Fig. 9). Response rates according to RECIST criteria were 31.6% and 9.4% for patients treated with FOLFIRINOX and gemcitabine respectively (p<0.001)[102].

The toxicity profile of FOLFIRINOX, a triplet chemotherapy, was less favourable than that of gemcitabine monotherapy, with higher rates of severe (grade 3 or 4) toxicities such as neutropenia (45.7% vs. 21.0%), febrile neutropenia (5.4% vs. 1.2%), thrombocytopenia (9.1% vs. 3.6%), diarrhoea (12.7% vs. 1.8%), and sensory neuropathy (9.0% vs. 0%), whereas the only grade 3-4 toxicity occurring more frequently in the gemcitabine arm was alanine aminotransferase elevation (7.3% vs. 20.8%)[102].

Nonetheless, the matter concerning increased toxicities with FOLFIRINOX and quality of life was dealt with in a subsequent paper by the same group[105], by means of further investigation of the patients included in the PRODIGE4/ACCORD11 trial. Achieving the best possible health-related quality of life should be one of the most significant purposes of treatment for patients with mPDAC because of the short life expectancy and the fact that patients are usually highly symptomatic when diagnosed. In this work, the investigators employed the European Organisation

for the Research and Treatment of Cancer Quality of Life Questionnaire C30 (EORTC QLQ-30) to assess the global health status, the level of functioning in various domains, and the severity of symptoms in 342 patients randomised to be treated with gemcitabine or FOLFIRINOX. The findings clearly showed that, despite the increased toxicities associated with FOLFIRINOX, there were no notable differences between the two arms concerning quality of life in the domains examined in the EORTC QLQ-C30, except for diarrhoea, which seemed to affect patients in the FOLFIRINOX group predominantly in the first two months of treatment. Notably, time until definitive deterioration (the time span after which QLQ-C30 score decreased by more than 20 points compared to the baseline score) was significantly higher for the FOLFIRINOX arm as for global health status, physical, role, cognitive, and social functioning, and six symptom domains (fatigue, nausea/vomiting, pain, dyspnea, anorexia, and constipation). These findings demonstrated that a clinically meaningful prolongation of quality of life can be achieved adopting FOLFIRINOX as opposed to gemcitabine in patients fit for polychemotherapy, in spite of the less favourable safety profile of this regimen[105].

After more than a decade of unsuccessful randomised trials aiming to add another cytotoxic agent to gemcitabine, the MPACT trial, whose results were published in 2013, demonstrated the efficacy of gemcitabine plus nab-paclitaxel in this setting[103].

Nab-paclitaxel (nanoparticle albumin-bound paclitaxel) is a solvent-free colloidal suspension of the taxane and human serum albumin. This agent targets one of the main factors which causes pancreatic cancer to be resistant to chemotherapy: the fact that cytotoxic drugs fail to reach high concentrations within the tumour tissue. This is due to the desmoplastic reaction of the stroma, which causes a rise in interstitial fluid pressure, which, in turn, impairs transport of molecules from the vessels to the tumour tissue, diminishing the delivery of chemotherapeutic agents to malignant cells[106]. The secreted protein acidic and rich in cysteine (SPARC), a multifunctional glycoprotein overexpressed in pancreatic adenocarcinoma, modulates the malignant cells-ECM interaction, plays a pivotal role in tumour growth and migration, promotes angiogenesis, and is probably involved in

tumour escape by inhibiting immune surveillance. SPARC binds albumin and has been hypothesised to mediate the transport of albumin from the ECM to tumour cells. Nab-paclitaxel takes advantage of albumin as a carrier for hydrophobic molecules in the blood and exploits its transcytosis across vascular endothelium; albumin's binding to SPARC allows then the intratumoral accumulation of this drug[106]. Moreover, nab-paclitaxel has been found to increase the concentration of gemcitabine in tumour cells by reducing the levels of cytidine deaminase, the primary enzyme involved in gemcitabine metabolism, through reactive oxygen species-mediated degradation[107].

The MPACT trial was an international, multicenter, phase III study in which 861 patients with mPDAC were randomised to receive either gemcitabine alone or gemcitabine plus nab-paclitaxel[103]. In both arms, chemotherapy was administered weekly for 7 out of 8 weeks (first cycle), whereas in the subsequent cycles gemcitabine or gemcitabine plus nab-paclitaxel were given weekly for 3 every 4 weeks. Nab-paclitaxel was administered in a 30-to-40-minute intravenous infusion at a dose of 125 mg/m^2, followed by a 30-minute infusion of 1000 mg/m^2 of gemcitabine.

Eligible patients had histologically confirmed mPDAC, had not previously received chemotherapy for advanced disease and were required to have adequate hematologic, hepatic, and renal function and a Karnofsky performance status of 70% or more.

The primary endpoint of the study by von Hoff and colleagues was overall survival, while progression-free survival and response rate (RR) were secondary endpoints. The median OS was significantly longer in the gemcitabine plus nab-paclitaxel group, at 8.5 months, as opposed to 6.7 months with gemcitabine only (hazard ratio for death, 0.72; 95% CI, 0.62 to 0.83; $p<0.001$). Similarly, patients treated with gemcitabine plus nab-paclitaxel had a significantly longer PFS, with a median of 5.5 months, compared to 3.7 months for patients treated with gemcitabine alone (hazard ratio for disease progression or death, 0.69; 95% CI, 0.58 to 0.82; $p<0.001$) (Fig.10). The RR of the new regimen was more than triple that of gemcitabine: 23% vs. 7% ($p<0.001$)[103].

Gemcitabine plus nab-paclitaxel has a relatively favourable safety profile, with an only slightly

higher proportion of patients facing severe (grade 3 or 4) toxicities (50% vs. 43%) compared to gemcitabine monotherapy. Grade 3 or higher adverse effects reported more often in the gemcitabine plus nab-paclitaxel group included neutropenia (38% vs. 27%), fatigue (17% vs. 7%), and peripheral neuropathy (17% vs. 1%)[103].

No randomised clinical trial directly compared gemcitabine plus nab-paclitaxel and FOLFIRINOX. Cross-trial comparison is not entirely reliable: the selection criteria for patients in the two above-mentioned studies were somewhat different, e.g. patients aged over 75 were excluded from the PRODIGE4/ACCORD11 study, while more than 10% of the patients included in the MPACT trial were aged 75 years or older, the eldest being 88 years old. Nonetheless, available data would suggest that FOLFIRINOX is more active, but also more toxic[87].

Current guidelines, in the absence of a head-to-head comparison, recommend both FOLFIRINOX and gemcitabine plus nab-paclitaxel for the treatment of patients with mPDAC whose ECOG PS is 0 or 1, whose bilirubin level are below 1.5 x ULN and who have a favourable comorbidity profile (i.e. normal or subnormal blood work values, liver and kidney function, and absence of comorbid medical conditions requiring active ongoing medical care, such as congestive heart failure, chronic obstructive pulmonary disease, uncontrolled diabetes mellitus, and neurological disorders)[67,108]. It is worth noting that ASCO recommends FOLFIRINOX for patients with a "favourable" comorbidity profile, while gemcitabine plus nab-paclitaxel is recommended for patients who have a "relatively favourable" comorbidity profile[108], suggesting the adoption of this less toxic, but probably less effective regimen, for the first-line treatment of patients who are fit for polychemotherapy, but whose global health status is slightly worse than that of patients eligible for FOLFIRINOX.

No more than half patients presenting with metastatic disease at diagnosis are suitable for aggressive chemotherapy. For patients with ECOG PS 2 and/or bilirubin levels > 1.5 x ULN, monotherapy with gemcitabine is appropriate, while patients with significant uncontrolled comorbidities, a very poor performance status (ECOG PS 3 or 4), or a presumably very short life

expectancy are not eligible for cancer-directed therapy and should be treated with best supportive care only[67,108].

A number of targeted therapy agents have been tested in mPDAC, but the results of all phase III trials have been negative. The only exception was the combination of gemcitabine and the EGFR tyrosine kinase inhibitor erlotinib, which obtained a 12-day improvement in median OS[109]. Albeit statistically significant, this is highly likely to be clinically irrelevant and to be achieved at the cost of increased toxicity. As a consequence, despite the approval of this doublet, this regimen has not been widely used in the routine clinical activity.

A subgroup of patients with good performance status after the failure of first-line treatment could benefit from second-line chemotherapy. No randomised clinical trial to date has investigated second-line therapy after FOLFIRINOX or gemcitabine plus nab-paclitaxel. According to the most up-to-date ASCO guidelines, gemcitabine plus nab-paclitaxel could be offered as second-line treatment after FOLFIRINOX, while 5-FU/LV plus oxaliplatin (FOLFOX), irinotecan (FOLFIRI), or nanoliposomal irinotecan could be used after first-line therapy with gemcitabine plus nab-paclitaxel[108]. The only randomised trials whose results are available concern mPDAC progressed on first-line gemcitabine. The CONKO-003 phase III trial compared a 5-FU/LV plus oxaliplatin (OFF regimen), with 5-FU/LV alone for gemcitabine-refractory mPDAC, showing a longer OS for patients treated with the combination regimen (5.9 months vs. 3.3 months)[110]. However, the analogue and more recent PANCREOX trial failed to confirm these results[111]. Last year, the outcomes of the NAPOLI-1 phase III trial were published: 5-FU/LV plus nanoliposomal irinotecan was reported to achieve better OS (6.1 months vs. 4.2 months), PFS (3.1 months vs. 1.5 months), and RR (16% vs. 1%) than 5-FU/LV alone[112]. According to the current ESMO guidelines, considering the inconsistent results regarding the use of oxaliplatin, 5-FU/LV plus nanoliposomal irinotecan may become the best option for second-line chemotherapy in patients previously treated with gemcitabine[67].

All in all, despite the significant advances of the last years, the prognosis of patients who present

with metastatic disease and are treated with the newest chemotherapy regimens is still grim, with a median survival inferior to 12 months.

Chapter 2. Circulating tumour DNA: the "liquid biopsy".

2.1. Accessing tumour DNA via tissue sampling: biopsy and related issues

Alterations in the genome of malignant cells are highly informative in clinical oncology. Specific mutations, for instance, can act as prognostic or predictive markers. For example, positivity for *Her-2/neu* amplification in breast cancer is a marker of worse prognosis and predicts response to anti-Her-2 antibodies such as trastuzumab or pertuzumab[113], while *KRAS*, *NRAS* and *BRAF* mutations in colorectal cancer predict resistance to therapy with the anti-EGFR monoclonal antibodies cetuximab and panitumumab[114–116].

Targeted therapies usually rely on the acquisition of tumour tissue for molecular analysis before initiation of treatment. Tissue sampling by biopsy or surgical excision is the gold standard both for histological examination with the purpose of making a conclusive diagnosis and for the molecular analysis of specific cancer-associated genomic aberrations. Apart from the ones mentioned above, clinical applications include detection of *EGFR* mutations for treatment with EGFR tyrosine kinase inhibitors (TKIs) such as erlotinib and gefitinib[117] and *ALK* mutations for therapy with crizotinib in non-small cell lung cancer[118], *BRAF* mutations for BRAF inhibitors (e.g. vemurafenib, dabrafenib) in melanoma[119,120], and *c-KIT* mutation to initiate imatinib in gastrointestinal stromal tumours[121]. In pancreatic cancer, however, targeted therapy has proven of little use, the only targeted agent approved being erlotinib in association with gemcitabine[109], a far less active treatment than FOLFIRINOX or gemcitabine plus nab-paclitaxel.

Tissue sampling via biopsy is a technique that poses major issues: biopsies are an inconvenience from a scheduling perspective, possibly delaying the initiation of treatment, are highly expensive, and are invasive, painful manoeuvres whose results may not influence the outcome. Last, but not least, biopsies are not without risks. A work conducted at MD Anderson Cancer Center reported adverse event rates of 17.1% and 1.6% respectively for thoracic and abdominal/pelvic sampling[122]. Concerns have also been raised regarding very rare, but possible tumour seeding along the needle

tract[123]: for example, transscrotal biopsy in testicular cancer is not recommended[124], because malignant cells could be left in the scrotum and spread to inguinal lymph nodes.

The number of malignant cells in each biopsy varies and is largely dependent on the tumour cellularity and size of the specimen acquired; fine-needle aspirates, for instance, only allow the collection of a very limited amount of tumour tissue in comparison with surgically resected specimens.

Another important matter is sample preservation. Most tumour biopsies are preserved in the form of formalin-fixed paraffin-embedded (FFPE) blocks; during fixation with formalin, a number of chemical reactions occur, including DNA denaturation, fragmentation and introduction of nonreproducible sequence alterations. The majority of point mutations in the genome of cells contained in an FFPE block are C:G > T:A changes as a consequence of cytosine deamination to uracil[125]. As a result, formalin fixation does not allow adequate preservation of high-molecular-weight DNA, with up to 30% of nucleic acids possibly lost during fixation. A review of mutation detection failures indicated an 11.9% rate of missed mutations; 80% of these are attributable to pre-PCR error[126].

Another major limitation of tissue biopsy is tumoral heterogeneity, a feature of most advanced cancers[127]. Malignancies are dynamic, with different genetic profiles (type and proportion of specific genetic alterations) to be found in various areas of the same tumour - i.e., intratumoral heterogeneity; the same is applicable for different metastases within the same patient - i.e., intermetastatic heterogeneity[128]. A biopsy can only sample tissue from a zone of one selected lesion, thus missing the intratumoral and intermetastatic heterogeneity. Moreover, a biopsy is a single snapshot of the evolution of the sampled area of the tumour in time[129]: our knowledge of the genetic composition of the tumour as a whole is therefore drastically limited, and, as a result, the factors causing primary or secondary resistance often remain unknown.

Due to genomic instability, tumours are in constant evolution, and new clones arise during progression. Medical therapy adds a selection pressure: resistant subclones emerge as a result of

additional aberrations in the pathways related to the mechanism of action of the agent used. In malignancies in which patients diagnosed even in advanced stages enjoy a relatively long survival, such as breast or prostate cancers, the molecular analysis of biopsies conducted many months (or even years) before, during which a number of lines of treatment have been administered, may very possibly be inadequate to understand the biology of the tumour at the time of initiating a new therapy[130]. Although rebiopsying a selected lesion at any therapeutic decision point could seem a solution to this issue, because of the invasiveness and morbidity associated with the procedure, molecular analysis is usually performed on archival tumour tissue[129,130].

In conclusion, a biopsy can be a difficult, painful and potentially harmful procedure, is a single photograph of the tumour in time, and is subject to selection bias as a result of intratumoral and intermetastatic heterogeneity. It is thereby clear that new procedures through which to analyse the genetics of cancer are urgently needed. These new techniques should have the following features: be rapid, cost-effective, be less invasive than a biopsy, provide a picture of all the genetic patterns present in a malignancy, and allow the clinician to keep up with the genomic alterations that occur in cancer cells exposed to therapy.

2.2. The liquid biopsy

The "liquid biopsy", i.e. the extraction, quantification, and characterisation of circulating tumour cells or circulating tumour DNA (ctDNA) from a sample of peripheral blood, is a very intriguing procedure in the respect mentioned above.

During the history of an invasive carcinoma, malignant cells overstep the basal membrane and invade the underlying connective tissue, where they can intravasate in blood and lymphatic vessels. Tumour cells free in the peripheral blood, possibly on their way to colonise distant organs forming metastases, are known as circulating tumour cells (CTCs) and have been the subject of extensive investigation during the past few decades. CTCs have been proven to be a prognostic marker in a number of tumours, including breast, colorectal, prostate and lung cancer[131–133].

The analysis of cell-free circulating tumour DNA is another possible technique that may supplement or replace tissue biopsies. DNA can be isolated from the cell-free portion of peripheral blood as low-molecular-weight fragments (<1000 bp). As the procedure is only minimally invasive (venipuncture) for the patient and the testing is relatively inexpensive, the liquid biopsy can be repeated many times. This would enable longitudinal monitoring of the patient and the acquisition of actionable, real-time information on the current genetic profile of the tumour whenever a new therapy must be initiated[134–136].

2.3. Circulating tumour DNA: biological aspects

The presence of cell-free nucleic acids in the blood was reported for the first time by Mandel and Metais[137] in 1948 but went overlooked for at least a couple decades, when Tan et al. found that both free DNA and anti-DNA antibodies could be detected in the serum of patients with systemic lupus erythematosus[138].

Circulating cell-free DNA (cfDNA) has been the subject of extensive research in a number of clinical fields, such as stroke, myocardial infarction, end-stage kidney failure, transplants, surgery, trauma, and pregnancy, showing that cfDNA can be detected at a basal concentration in a steady-state context, and its levels soar in case of cellular injury or necrosis[134].

The first investigators to report findings suggesting the potential interest of cfDNA in the oncologic setting were Leon and co-workers[139] in 1977, who described elevated levels of cfDNA in approximately half the members of a group of 173 patients undergoing radiotherapy for several different malignancies. Patients with metastatic disease were found to have greater amounts of DNA in their serum, compared to those with localised disease; DNA levels decreased remarkably after radiotherapy, with persistently high or rising cfDNA concentrations associated with a lack of response to treatment.

Even more intriguingly, in 1989, Stroun et al.[140] showed that circulating DNA in the plasma of oncologic patients shared biophysical properties (impaired strand stability) with DNA of malignant

cells, thus suggesting the origin of the increased levels of cfDNA from cancer cells themselves, instead of activated lymphocytes reacting towards the disease as an alternative theory would have implied.

Moreover, circulating tumour DNA (ctDNA) can be discriminated from background cfDNA because the tumour genome, by definition, contains somatic genetic alterations. Several cancer-specific mutations in oncogenes and tumour suppressor genes have been detected in circulating DNA: the first were *NRAS* in patients with myelodysplastic syndrome and acute myelogenous leukaemia (AML)[141], rearranged Ig heavy chains in patients with a B-cell malignancy such as non-Hodgkin's lymphoma or acute B-precursor lymphoblastic leukaemia[142], and *KRAS* mutations in colorectal[143] and pancreatic cancer[144].

The mechanism through which tumour cells shed fragmented DNA into circulation has still to be ultimately determined. It has been suggested that all vital cells actively secrete DNA into the bloodstream[145]. In a study published in 2001, instead, Jahr et al.[146] hypothesised that ctDNA originates from apoptotic and necrotic cells. The predominant form of circulating DNA is a structure known as mononucleosome, in which a fragment of DNA of 180-200 bp is associated with proteins; this is the result of internucleosomal cleavage of chromatin, a major feature of apoptosis, thus suggesting this process plays a pivotal role[134,147]. Circulating tumour DNA (ctDNA) is believed to be released from neoplasms primarily as a result of necrotisation, while non-tumour cfDNA would originate from normal cells which undergo apoptotis[148]. In fact, what happens during the growth of a neoplasm is that cellular turnover increases and more and more cells die from apoptosis and necrosis: while their remains are effectively cleared by macrophages under physiologic circumstances, within the mass cellular debris accumulates and is later released into the bloodstream (Fig. 11)[134]. For healthy subjects, the average concentration of cfDNA was found to be around 10-30 ng/ml, while values for oncologic patients often exceed 100 ng/ml[136].

Malignancies composed of about 50 million cancer cells release enough DNA for the detection of ctDNA in blood; in contrast, usually, imaging can recognise masses of approximately 7–10 mm

in size, containing roughly 1 billion cells[135].

The contribution of cancer cells to total cfDNA is highly variable: the studies investigating this aspect have highlighted proportions ranging from 0.01% to 90% and more[146,149]. The passive release of ctDNA from apoptotic or necrotic cells within the tumour depends on the site, size, and vascularity of the mass, possibly explaining the high degree of variation in ctDNA concentrations and mutant fractions[134].

As ctDNA often represent less than 1% of total cfDNA, with an absolute concentration ranging from 1 to 100 ng/ml, its measurement can be challenging. Early studies would use both serum and plasma to extract ctDNA: as during coagulation the lysis of leukocytes can occur, increasing the quantity of non-tumour circulating DNA, the plasma would seem to better reflect the in vivo levels of circulating DNA[147,150]. Also, the plasma may contain a higher concentration of ctDNA, as EDTA indirectly inhibits blood DNases[151].

It has been demonstrated that the whole tumour genome can be reconstructed from its small circulating fragments; the fact that whatever DNA segment contained in the cells of the tumour can potentially be found in ctDNA justifies the term "liquid biopsy"[129].

There are few data available concerning the stability of ctDNA in the blood: clearance seems to be rapid, with a half-life of less than 2 hours, and the spleen, liver, and kidneys could be the responsible organs[129].

The analysis of ctDNA can be performed with two possible approaches. The first one targets known genetic alterations of the tumour, such as mutations in *EGFR* and *KRAS*. The detection of mutant alleles in very low proportions is nowadays possible thanks to highly sensitive technologies such as ARMS (amplification refractory mutation system), digital PCR (dPCR), and beads, emulsions, amplification, and magnetics (BEAMing)[129]. A number of assays for driver mutations which hold implications for therapeutic decision-making exist. The other possible approach, instead, is untargeted and, therefore, does not require previous knowledge of the genetic changes specific to the tumour. A genome-wide analysis of the liquid biopsy is carried out thanks

to next-generation sequencing (NGS) techniques[129], which, however, have a lower sensitivity than the above-mentioned methods (Figure 12)[134].

A liquid biopsy with ctDNA would have a number of benefits, compared to tissue biopsy (Table 5). First and foremost, it is a minimally invasive method to analyse tumour genome, thus avoiding the possible adverse events of tumour tissue sampling and enabling repetition at multiple time points. Consecutive liquid biopsies would provide, in a way, a "video" of the evolution of the molecular profile of the tumour, instead of relying on the single photograph given by the conventional biopsy. Secondly, given the fact the ctDNA is released into the bloodstream from all the sites of disease, both the primary tumour and distant metastases, intra-tumoral and inter-metastatic heterogeneity are adequately taken into account by liquid biopsy, while tissue biopsy is suboptimal in this respect. Lastly, the blood is a source of fresh DNA, not altered by fixatives.

In a number of studies it has been investigated if the detection of cancer-specific mutations would be consistent in tissue specimens and liquid biopsies: sensitivity and specificity have generally been satisfactory. A French study was the first to report multiplexed detection of targetable mutations in a population of patients with metastatic cancer (multiple histological types) included in a prospective trial[152]. Molecular analysis of tissue biopsy and liquid biopsy had minimal discrepancy: 29 mutations were identified in the tissue samples, of which 28 were found in the liquid biopsies as well (sensitivity: 97%), and one additional mutation in a liquid biopsy only. This latter finding is quite frequent and is usually interpreted as a consequence of the fact that tumour heterogeneity is effectively dealt with by ctDNA analysis.

2.4. Circulating tumour DNA as a new tumour marker

Liquid biopsy with ctDNA could be adopted for a bunch of different purposes in clinical activity. Possible applications include its adoption as a novel biomarker, similarly to protein tumour markers already employed in the clinical practice, to monitor response to treatment, precociously detect progression, identify minimal residual disease, evaluate molecular heterogeneity, or provide

an early diagnosis of disease[135]. In other words, ctDNA has the potential to become a diagnostic, predictive, and prognostic marker in the management of cancer (Table 6).

2.4.1. Monitoring of treatment response and tumour burden

Disease burden is usually assessed with imaging; serum biomarkers such as PSA, CEA, CA-125, CA15-3 and CA19.9 can be helpful in evaluating response to treatment, particularly when it may be difficult to discriminate between the tumour and surrounding tissue reactions and imaging results could be inconclusive. However, many cancers lack a reliable circulating protein marker, and these often have suboptimal sensitivity and specificity, with a substantial proportion of false negatives and false positives. For instance, as mentioned above, serum CA19.9 levels can increase because of various non-malignant pancreatobiliary conditions in which the biliary excretion of the antigen is impaired[50]. Furthermore, the half-life of protein markers is usually in the order of weeks; as a result, a similar time span between measurements is required, and months can be necessary to see a reliable trend[134].

A comparison of protein tumour markers with ctDNA clearly shows some pros of the latter. ctDNA has high specificity: somatic cancer mutations are, by definition, present in tumour DNA and absent in wild-type, normal DNA; this avoids the occurrence of false positives as for imaging and protein markers. The short half-life of ctDNA (according to Diehl et al.[149], just under 2 hours) allows a much more frequent assessment of tumour dynamics. As a result of the shorter half-life, ctDNA has a notable depth of response, too, with changes in ctDNA reported to anticipate variations in protein marker levels and radiological responses by weeks or even months[149,153].

In a way similar to viral load changes (e.g. HIV, HBV, HCV viral load), rises and falls in ctDNA correlate with the clinical course of the disease. As regards patients with advanced tumours receiving medical therapy, works concerning several malignancies have investigated this potential application of ctDNA to monitor treatment response: colorectal cancer (CRC)[149], gastric cancer[154], melanoma[155–157], breast cancer[153] and non-small cell lung cancer (NSCLC)[158,159]. Findings have

generally been consistent: progression of disease is associated with rapid increases in ctDNA levels while successful medical therapy and surgery correspond to drops in ctDNA levels[134].

Intriguingly, Diehl and co-workers[149] reported that no patient with undetectable ctDNA after surgery for colorectal cancer experienced recurrence of disease, while this was the case for all but one of the subjects with detectable ctDNA after resection surgery within a year. High concentrations of plasma mutant *KRAS* were proven to be negative prognostic markers in patients with metastatic colorectal cancer[160,161].

A Japanese study focusing on disease recurrence after gastrectomy for gastric cancer indicated that circulating mutant *TP53* levels, and not total cfDNA concentration, showed a good correlation with the disease status, decreasing remarkably after radical (R0) surgery and less so after R1 and R2 resections, and increasing at recurrence.

Shinozaki and colleagues[155] found that in a group of 20 patients with advanced melanoma treated with polychemotherapy and immunotherapy, after treatment serum mutant *BRAF* was detectable in only 10% of the patients who responded to biochemotherapy, while it was measurable in 70% of non-responders. It was also pointed out that the detection of serum mutant *BRAF* is associated with shorter overall survival. In a study by Lipson et al.[156], twelve patients undergoing treatment with immune checkpoint blockade were tracked using specific ctDNA hotspot mutations in *BRAF*, *cKIT*, *NRAS* and other genes; ctDNA variations correlated with clinical and radiological outcomes. Sanmamed et al.[157] instead reported that in a group of 20 patients with *BRAF* V600E mutation treated with vemurafenib or dabrafenib baseline plasma mutant *BRAF* correlated with tumour burden and was associated with poorer PFS and OS, and ctDNA levels increased at disease progression compared to best response.

Dawson et al.[153] successfully detected ctDNA in 29 out of 30 patients with breast cancer with pre-determined genetic alterations (point mutations in *PIK3CA* and *TP53*, structural variants) at molecular analysis of archival tissue specimens. The authors indicated that ctDNA, compared to CTCs and CA15-3, had a greater dynamic range and better correlation with changes in tumour

load as seen on imaging. In 53% of the patients with progressive disease, increasing levels of ctDNA were detected on average 5 months (range 2-9 months) before progression of disease was confirmed radiologically; increasing levels of ctDNA correlated with inferior overall survival.

Given the dramatic effects of EGFR-TKIs on *EGFR*-mutant NSCLC and the fact that there are not so highly prevalent mutations in oncogenes and tumour suppressor genes in NSCLC as, for instance, in pancreatic and colorectal cancer, research has been focusing mainly on mutant *EGFR* ctDNA. In a study by Imamura et al.[159] about *EGFR*-mutated patients treated with EGFR-TKIs, ctDNA responses during therapy correlated well with radiological changes in radiological good responders, while the association was marginal in non-responders. The investigators speculated that tumours which had a complete/partial radiological response would be mostly composed of cells with activating *EGFR* mutations, while patients with stable disease or progression of disease but positive ctDNA responses might have a tumour which is a mixture of wild-type *EGFR* and mutant *EGFR* cancer cells. This study also addressed a very significant issue as regards liquid biopsy, the time span after which subsequent samples should be collected after baseline assessment. The investigators reported that early (< 1 month) ctDNA levels were not good predictors of radiological response to treatment, because ctDNA levels could soar early after the initiation of medical therapy due to rapid tumour necrosis. Another interesting outcome was that in the 11 patients out of 17 with radiologically confirmed progression in which *EGFR* ctDNA levels increased during therapy, ctDNA changes anticipated radiological findings by an average of 4 weeks. An elevated *EGFR* T790M ctDNA concentration was detectable in 52% of patients experiencing progression of disease: T790M is the main secondary mutation causing resistance to EGFR-TKIs, present in 50-60% tumours progressing after first-line biological therapy. New agents such as osimertinib and rociletinib are active in T790M *EGFR*-mutated NSCLC, and their efficacy has been shown in patients with a liquid biopsy positive for this mutation[162]. Another study had similar results, confirming that dynamic changes in *EGFR* ctDNA relative to baseline may predict response to treatment.[158]

In conclusion, data available in several malignancies suggest that ctDNA could be a useful biomarker to supplement imaging and protein markers to evaluate tumour load and treatment response.

2.4.2. Monitoring of tumour heterogeneity and resistance to targeted therapy

ctDNA could also be used as a predictive marker for biological therapy. For instance, in CRC, mutant *KRAS* is a predictive marker for resistance to EGFR blockade, while in NSCLC mutations in *EGFR* predict response to therapy with EGFR TKIs. Patients often develop secondary resistance to these targeted agents within some months of treatment. Liquid biopsy could be performed to examine the status of the predictive genetic determinants instead of tumour section analysis. The liquid biopsy would allow to bypass the issue of repeated posttreatment tumour tissue sampling, as well as providing a better overall picture of the genetic status of the malignancy at multiple time points (Fig. 13).

Several works have investigated the feasibility of this approach in CRC. A study reported 92% sensitivity and 98% specificity for seven tested 7 *KRAS* mutations in ctDNA, with a concordance rate 96% compared to tumour specimen examination[163]. Other works indicated that 38% of patients treated with panitumumab developed detectable *KRAS* mutations in circulating DNA within 5-6 months of treatment[164], and the presence of mutant oncogenes in circulation preceded radiological progression by an average of 10 months[165].

In NSCLC, instead, several works reported sensitivity and specificity of liquid biopsy for *EGFR* mutations compared to tissue analysis of 65-75% and 93-100% respectively[158,166–168]. Overall, available data indicate that *EGFR* mutation detection in ctDNA could be used as a useful surrogate of tissue-based testing to predict the clinical outcomes of EGFR TKIs therapy, especially if tumour tissue is not available or the sample is exhausted[158,167,168].

The real-time assessment of genetic markers predictive of sensitivity or resistance to targeted therapy in the future will enable the clinician to switch towards alternative treatments or

combinational regimens suppressing the expansion of clones potentially accountable for the failure of treatment[134,135].

2.4.3. Monitoring of minimal residual disease

Another potential field in which ctDNA could prove useful is the adjuvant setting, after curative-intent surgery. For example, in localised CRC or breast cancer, resection surgery alone cures a large proportion of patients. However, at the moment we cannot determine which patients have been cured and which have a subclinical residual disease that will result in disease recurrence. As a consequence, patients with high-risk clinical and pathologic (TNM staging) criteria indiscriminately undergo adjuvant chemotherapy; however, a substantial fraction of these subjects do not need such potentially toxic therapy. ctDNA could serve as a marker of minimal residual disease after surgery, identifying the patients who have a high risk of recurrence (Fig. 14). High sensitivity will be needed to detect the low levels of ctDNA released from micrometastases not detectable by imaging. Tissue damage during surgery causes ctDNA levels to rise; as a result, ctDNA should be assessed 6-8 weeks after surgery, before the initiation of adjuvant therapy[134,135].

Diehl et al.[149] examined a cohort of patients with CRC who underwent curative-intent resection. Eighteen patients were followed up for 2 to 5 years. This study showed that ctDNA could be sensitive enough to detect minimal residual disease after surgery. Virtually all patients with detectable postoperative ctDNA experienced recurrence of disease; in contrast, all patients with undetectable ctDNA after surgery did not relapse.

Tie et al.[169] have presented at the 2016 ASCO Annual Meeting a prospective trial in 231 patients with stage II CRC, who had undergone surgery and were followed up with a liquid biopsy every 3 months, starting 4-10 weeks after resection. 79% of patients with positive ctDNA who received no adjuvant treatment experienced recurrent disease, while only 10% of patient with undetectable ctDNA who did not receive adjuvant chemotherapy relapsed. Among subjects who received adjuvant treatment, ctDNA was detected in 6 patients early after resection; in all of them, the

ctDNA status turned from positive to negative during chemotherapy but became positive again after completion of adjuvant treatment in 2 patients, who both went on to develop a relapse. Considering all patients with several liquid biopsies available, ctDNA detection anticipated radiological recurrence by an average of 167 days. The authors proposed that ctDNA could reshape the design of clinical trials in the adjuvant setting, as ctDNA analysis allows the clinician to identify a priori patients with minimal residual disease, who, therefore, are at high risk of relapse, and also serves as a marker of response to adjuvant chemotherapy.

ctDNA has also been used in a study focusing on 20 patients with breast cancer to assess recurrence after neoadjuvant therapy and resective surgery[170]. The primary tumour was analysed using NGS and mutations were identified in 60% of the tumours; tailored digital PCR assays were then used to evaluate ctDNA during follow-up. ctDNA was detected in 75% of samples before neoadjuvant therapy, with no difference to be found between patients with and without recurrence of disease. Four out of the five patients with recurrent disease had specific ctDNA detectable in the first six months post-surgery; all the patients with detectable ctDNA relapsed and no patients who did not experience recurrence of disease had detectable ctDNA post-surgery. The negativity of ctDNA after surgery thus indicated total clearance of the tumour. Remarkably, a patient with brain recurrence did not have detectable ctDNA at relapse.

Taken together, these studies would suggest that ctDNA could be highly valuable in the management of patients who underwent curative-intent surgery, revealing those patients who have residual disease and providing evidence of the efficacy of adjuvant treatment.

2.4.4. Circulating tumour DNA for early diagnosis of cancer

As regards the potential employment of circulating DNA as a diagnostic tool, the first approaches concerned the concentration of total cfDNA in cancer patients as opposed to healthy control subjects. Although several works showed that oncologic patients have significantly higher levels of cfDNA, thus enabling the confirmation of cancer diagnosis or, vice versa, the

identification of disease-free patients after curative-intent treatment[171,172], others pointed out that the discriminatory power of such a test would be very low[173,174]. Therefore, cfDNA alone without determination of cancer-specific mutations is of little use in this respect.

Instead, the development of assays for aberrations which are recurrent in cancers would seem promising for early detection of cancer, because of the exquisite specificity of ctDNA and the day-by-day increasing sensitivity of the techniques involved. The available evidence suggests that benign tumours and nonneoplastic conditions do not release ctDNA in the bloodstream[175], so false positives are unlikely. A positive result of a screening test with ctDNA should raise concern, because of the known causal relationships between the examined mutations and cancer. Nowadays, inclusion criteria for clinical trials usually include the histological/cytological demonstration of the presence of the tumour; in the future, clinical trials could allow the inclusion of patients who have somatic mutations in circulating DNA.

Bettegowda and co-investigators[176] examined 410 patients with various cancers, reporting more than 80% of patients with metastatic disease to have detectable levels of ctDNA, a higher proportion than that of most conventional markers[177]. A notable exception were malignancies arising in the central nervous system, which did not release detectable quantities ctDNA in the bloodstream as frequently: for example, only 10% of patients with glioma were positive for ctDNA[176].

The same study took into consideration patients with localised disease, showing detectable ctDNA in 73, 57, 48 and 50% of patients with CRC, gastroesophageal cancer, pancreatic cancer and breast cancer respectively[176]. Additional findings were that the proportion of patients with detectable ctDNA and the ctDNA concentrations increased with stage. These data demonstrate that ctDNA is present in a large proportion of patients with this tumour types in stages I-III and this may thus be a useful diagnostic test for early detection of disease.

A recent advancement is the development of a blood-based screening test for CRC, which uses the methylated septin 9 gene (*SEPT9*) in plasma as a biomarker: this assay has been reported to

specifically identify the majority of CRCs of all sites and stages[178]. Septin 9 protein plays an essential role in pseudopod protrusion, tumour cell migration and invasion, and it has been shown that a region in its promoter is methylated in CRC. In malignancies with a viral aetiology, viral circulating nucleic acids could be employed for screening: circulating EBV DNA was used to diagnose early nasopharyngeal carcinoma[179].

2.5. Circulating tumour DNA in pancreatic adenocarcinoma

Yamada et al. were the first to detect circulating DNA harbouring cancer-specific somatic mutations in the plasma of patients with PDAC in 1998[180]. However, there are few studies in the literature concerning the analysis of ctDNA with the new, highly sensitive methodologies mentioned above in pancreatic cancer. Compared to other malignancies, PDAC has a momentous advantage when it comes to genetic alterations to be tested for in a liquid biopsy. More than 90% of pancreatic adenocarcinomas harbour mutations in the *KRAS* gene as founder mutations; as a result, detectable mutant *KRAS* fragments in cfDNA extracted from PDAC patients' blood imply the presence of circulating DNA originating from the tumour. Four point mutations, G12D, G12R, G12V, and G13D, account for 90% of all *KRAS* mutations in pancreatic cancer[181]. Consequently, it would not be strictly necessary to identify tumour-specific mutations in various oncogenes via molecular analysis of a biopsy specimen prior to ctDNA analysis, but the examination of a very limited number of mutations in one gene in a liquid biopsy would be sufficient. However, the concordance between mutations observed in cfDNA with those seen in PDAC tumour tissue is still to be conclusively determined.

The previously cited multi-disease study by Bettegowda et al.[176] included 155 patients with pancreatic cancer. The investigators reported the detection of ctDNA in approximately 85% of patients with advanced pancreatic cancer (n = 34) and 48% of patients with localised pancreatic cancer.

Earl et al.[182] examined cfDNA concentration and *KRAS* mutation detection in 31 patients with

PDAC. Despite using Droplet Digital PCR, one of the most sensitive technologies available, only 8 out of 31 (26%) patients had detectable mutant *KRAS* in cfDNA. These included 3 patients with resectable disease, one with LAPC and 4 with mPDAC. However, ctDNA *KRAS* positivity strongly correlated with overall survival in this study (60 days for *KRAS* mutation positive patients vs. 772 for *KRAS*-negative patients; hazard ratio for death 12.2, 95% CI 3.3-45.1, $p < 0.001$).

Sausen et al.[183] used digital PCR alongside with NGS of tumour tissue to identify cancer-specific mutations in 51 patients with resectable PDAC, largely focusing on alterations in the *KRAS* gene. The presence of ctDNA in plasma was demonstrated in 22 patients (43%), a similar proportion to the one reported by Bettegowda and co-workers. Specificity was > 99.9%. These findings suggest that a substantial proportion of early-stage PDACs could be diagnosed with this noninvasive method. The presence of ctDNA at diagnosis was predictive of disease recurrence ($p = 0.015$, log-rank test). Liquid biopsy was also repeated at several time points following resective surgery, revealing that patients with detectable ctDNA after surgery had a higher risk of relapse, compared to those with liquid biopsy negative for ctDNA ($p = 0.02$, log-rank test). The detection of ctDNA in plasma anticipated radiological determination of progression by an average of 6.5 months (3.1 months vs. 9.6 months after surgery, $p < 0.001$, paired t-test). These further results indicate that ctDNA analysis could be a highly performing test to monitor residual disease after resection and allow early detection of relapse.

The largest study investigating ctDNA in pancreatic cancer, which analysed blood samples from 259 patients, was published by Takai et al.[181] in 2015. Mutant *KRAS* was detected by Droplet Digital PCR in the plasma of 58.9% of patients with stage IV disease, 18.2% of LAPC patients, and 8.3% of patient with resectable disease. The proportion of ctDNA in total cfDNA ranged between 0 to 87.7%. Among the 9 *KRAS*-positive patients who underwent resection, 5 patients relapsed within 6 months after surgery, and had a poor prognosis. The authors suggested that patients with borderline resectable PDAC and mutated *KRAS* gene detected in plasma might be spared the risk of upfront surgery and be offered neoadjuvant chemotherapy. Multivariate analysis showed the

presence of mutant *KRAS* in plasma to be an independent negative prognostic factor for overall survival. The NGS Illumina method was subsequently used, finding comparable mutant *KRAS* frequencies. The investigators proposed that the reason why only a minority of patients with stage I-III pancreatic cancer had detectable mutant *KRAS* in plasma could be that PDAC is a hypovascular tumour and, therefore, little tumour DNA is released into the circulation as long as the disease is localised. Other authors claimed that the amount of wild-type stromal DNA released from the mass into the bloodstream could have a great impact on the relative amount of mutant circulating DNA[184]. Another reason might be that Droplet Digital PCR could not detect ctDNA because of the low quantity of template DNA in the small volume of plasma available for analysis; it may be possible to detect early-stage PDAC via liquid biopsy by using a larger volume of plasma (approximately 5 ml in the studies by Bettegowda et al.[176] and Sausen et al.[183]).

Kinugasa et al.[185] examined two cohorts of patients with pancreatic cancer. In the first one, *KRAS* mutations were determined both in EUS-FNA specimens and in cfDNA. *KRAS* mutations were detected in 74.7% of the tissue samples, while the percentage of patients with mutant *KRAS* in serum as determined by Droplet Digital PCR was 62.6%. The most frequently found mutation was G12D (38.6%), followed by G12V (34.6%) and G12R (5.3%). The concordance rate of *KRAS* mutations between EUS-FNA tissue specimens and ctDNA was 77.3%. In the second group, mutant *KRAS* ctDNA was detectable in 54.5% of patients. In both cohorts, Kaplan-Meier analysis demonstrated that median survival was significantly lower in patients with mutant *KRAS* ctDNA detectable (413 vs. 276 and 489 vs. 186 days respectively, $p < 0.02$), this aspect being particularly evident in the case of the G12V mutation. The authors speculated that ctDNA analysis could have the potential to become both a diagnostic and prognostic marker in pancreatic cancer. This study also used 20 individuals with chronic pancreatitis as controls; 4 subjects had detectable mutant *KRAS* in the absence of any evidence of pancreatic cancer. The presence of the mutated gene in cfDNA is likely to be transient and to originate from hyperplastic cells in the chronically inflamed pancreas. This finding is consistent with two other works, implying that mutant *KRAS* is not 100%

specific for PDAC[184].

Zill et al.[186] analysed ctDNA in blood compared to tumour biopsy samples in 17 patients with localised and advanced pancreatobiliary cancers. 90.3% of mutations detected in tumour biopsies were also found in cfDNA using NGS techniques. Assuming molecular analysis of the tissue biopsy as the gold standard, the diagnostic accuracy of cfDNA sequencing was 97.7%, with 92.3% average sensitivity and 100% specificity across five genetic determinants. The most commonly mutated genes were *KRAS* and *TP53*. Another aim of this was to test a correlation between dynamic changes in the mutant ctDNA proportion in cfDNA and disease course; eight patients were monitored with serial liquid biopsies and the conventional marker CA19.9 was determined concurrently. The mutant ctDNA proportion for the most prevalent mutation was compared to the CA19.9 concentration for each patient. The direction of change in CA19.9 and ctDNA percentage agreed significantly more often than by chance ($p = 0.02$). Moreover, changes over time in the ctDNA fraction of cfDNA correlated well with changes in CA19.9 (Pearson's $r = 0.69$ for interval slopes, $r = 0.93$ for interval differences). This finding suggests that ctDNA fraction changes reflect variations in tumour load during treatment in pancreatobiliary cancers.

Notably, all of the cited studies have been published within two years from the present time. These first data would classify ctDNA as a promising method for use as a liquid biopsy in pancreatic cancer.

ctDNA liquid biopsy could play a role in many different stages of pancreatic cancer management. Of note, obtaining a suitable tumour tissue specimen in pancreatic cancer is particularly problematic (up to 30% of biopsies are inadequate for diagnosis: QNS, quantity not sufficient, or TND, tumour not detected). This is because the anatomic position of the pancreas makes the procedure quite invasive, patients are often highly symptomatic and with a poor performance status, and neoplasms contain a high proportion of desmoplastic stroma[187].

Being able to use a simple peripheral blood sample instead would therefore be very appealing for diagnosis, given the minimal invasivity and the substantially lower costs, compared to EUS-

FNA: approximately US$50 vs. US$1405[188]. Also, histological examination and molecular analysis of a tissue biopsy are lengthy processes, while it only takes about 15 hours from receiving the plasma sample to obtaining final data using ctDNA analysis with Droplet Digital PCR[181]. Patients whose general conditions are too poor to undergo invasive tests, or those in which the results of the biopsy were inconclusive, would be the ones to benefit most from the liquid biopsy. However, in order to be implemented as a diagnostic tool, the capability of the liquid biopsy to detect ctDNA both in early-stage and advanced PDAC has to be definitively cleared[187].

ctDNA could be used to monitor patients after surgery: the findings of Sausen and co-investigators[183] about the opportunity to identify patients with residual disease and early detect relapse with the liquid biopsy need to be replicated in larger studies. In the future, it might be worth doing to look at whether initiating chemotherapy just after "liquid biopsy relapse" instead of waiting after radiological recurrence could have any clinical benefit[187].

In pancreatic cancer, assessing the efficacy of chemotherapy by CT or MR imaging is particularly difficult, because of the dense fibrotic tissue surrounding the tumour. Therapeutic decisions are, therefore, made taking into account CA19.9 levels. Theoretically, ctDNA could be used as a marker of response to treatment and disease progression detection just like CA19.9. However, there is little evidence for such an approach in patients with PDAC[187]. The majority of studies up to present day included a small number of patient and only collected a single blood sample to supplement data provided by molecular analysis of a tumour tissue specimen at diagnosis. To the best of the author's knowledge, the only work in which patients were prospectively followed-up with ctDNA interrogation during chemotherapy for locally advanced or metastatic PDAC is a small trial published earlier this year by Tjensvoll et al.[189]. In this study, plasma samples were collected from 14 patients prior to the initiation of treatment and subsequently every month during treatment. A peptide-nucleic-acide-clamp PCR was employed to detect *KRAS* mutations. The median follow-up time was 3.7 months (range 0.6-12.9 months). At baseline, 10 (71%) patients had detectable mutant ***KRAS*** in plasma, indicating the presence of ctDNA. Nine out of 10 experienced

progression of disease progression during follow-up, compared to only one (25%) of the four ctDNA-negative patients ($p = 0.01$). The pre-therapy ctDNA level was a statistically significant predictor of both PFS and OS ($p = 0.014$ and 0.010, respectively). An additional finding was that the ctDNA level at baseline was significantly higher in patients who experienced disease progression compared to patients with stable disease ($p = 0.024$) at follow-up. Kaplan-Meier survival analyses indicated trends towards shorter PFS and OS for patients who were ctDNA-positive before the initiation of chemotherapy ($p = 0.064$ and $p = 0.066$, respectively). For 10 out of the 14 patients, 2 or more follow-up samples were available; generally, ctDNA levels witnessed substantial variations during chemotherapy, which corresponded with CA19.9 levels and radiological responses in 3 patients, whose clinical history was extensively described by the authors. Comparing ctDNA levels and radiological responses, it would appear that ctDNA analysis may reveal disease progression before CT/MR scans, at least in a subgroup of patients. In conclusion, the results of this pilot study would seem to support the hypothesis that ctDNA has the potential to be used as a marker for predicting response to chemotherapy and reveal disease progression in advanced PDAC.

In the future, the analysis of liquid biopsies could provide a personalised genetic profile for patients with pancreatic cancer and identify which targeted agents could be most active. However, targeted therapy, with the possible exception of erlotinib, has still to be proven active in PDAC.

Imamura et al. wrote that "for the development of the field [liquid biopsy], pancreatic adenocarcinoma seems to be the ideal cancer to investigate"[190]. However, at present, it looks like we are only in possess of preliminary data and a great deal of further research is needed before achieving high-grade evidence on this topic.

Chapter 3. Our study: Circulating mutant *KRAS* as a biomarker to monitor response to treatment in pancreatic cancer

3.1. Rationale and aims of the study

At the present time, an optimal marker to monitor response to systemic treatment in advanced pancreatic cancer is still lacking. As a result of the above-mentioned difficulty to assess tumour volumes because of the desmoplastic stroma surrounding the tumour, the evaluation of treatment response in pancreatic cancer also takes into account serum markers. CA19.9 is the only approved circulating tumour marker for PDAC but has various limitations, such as suboptimal sensitivity (79%) and specificity (82%)[191]. These respectively result in a significant number of false negatives in the case of early invasive PDAC or preinvasive lesions, and false positives, which can occur, for instance, when the biliary excretion of the protein marker is diminished due to nonmalignant pathologies of the biliary tract (e.g. choledocholithiasis, cholangitis)[50]. Therefore, new circulating biomarkers to supplement information provided by imaging are urgently needed, so as to optimally manage these patients and improve their clinical outcome.

The detection of somatic mutations in cfDNA released in plasma is tumour-specific. Several studies have demonstrated that the ctDNA is measurable in blood samples collected from patients with both early and advanced pancreatic cancer[176,181–183,185,186,189]. ctDNA in advanced PDAC was reported to be detectable in up to 60-80% patients prior to systemic treatment[176,181,185,189].

KRAS is mutated in over 90% of PDACs; 4 point mutations, G12D, G12R, G12V, G13D encompass nearly all *KRAS* mutations in pancreatic cancer[181]. Thereby, we hypothesise that the longitudinal monitoring of *KRAS* somatic mutations in plasma may be instrumental for a better assessment of chemotherapy efficacy.

The primary endpoint of our proof-of-concept study is to correlate the levels of mutant *KRAS* in the plasma of patients with advanced pancreatic adenocarcinoma with radiological response to

treatment.

Secondary endpoints are to determine if and by how long ctDNA dynamic changes can anticipate the clinical response and if ctDNA positivity at baseline is predictive in terms of progression-free survival.

3.2. Materials and methods

3.2.1. Study population

24 patients with advanced pancreatic adenocarcinoma undergoing first-line chemotherapy were consecutively recruited at the Department of Medical Oncology of the Santa Chiara Pisa University Teaching Hospital from October 2015 to May 2016. 62 blood samples were collected between the beginning of the study and August 2016.

To be eligible, patients had to be fit for combination chemotherapy with regimens such as FOLFIRINOX or gemcitabine plus nab-paclitaxel. In other words, patients were required to have an ECOG Performance Status of 0 or 1 at the institution of chemotherapy and have normal or subnormal bilirubin levels, bone marrow, hepatic and renal function. Treatment was administered until disease progression, unacceptable toxicity or patient refusal.

Patients underwent standard disease monitoring by imaging according to Response Evaluation Criteria in Solid Tumours (RECIST) 1.1: a computed tomography (CT) or magnetic resonance imaging (MRI) scan of the abdomen and chest was performed at baseline and every 8-12 weeks. Tumour loads at baseline and at best response were calculated by adding up the diameters (in millimetres) of the RECIST 1.1 target lesions on the corresponding CT scan. Depending on the total, patients were classified as having or not a high subjective tumour burden, and as having a low, moderate or high tumour burden.

CA19.9 levels were determined at baseline and best radiological response. Data on performance status, adverse events, serum chemistry and haematology and concomitant medications were

collected at every chemotherapy cycle. Blood samples were drawn by venipuncture prior to the first cycle, after 14 days, 2 months and after radiological determination of disease progression.

Since the analysis of *KRAS* mutations in plasma is not part of the standard clinical management of PDAC, the study was submitted and approved by the Ethics Committee of Pisa University Hospital and conducted in agreement with the principles of the Declaration of Helsinki; all patients gave their signed informed consent before blood collection and ctDNA analysis.

3.2.2. Plasma collection and circulating cell-free DNA extraction

Six ml of blood were collected in an EDTA test tube and centrifuged at 4°C for 10 min at 3000 rpm within two hours after blood drawing. Plasma samples were stored at −80°C until analysis. Circulating cell-free DNA was extracted using a QIAmp® Circulating nucleic acid Kit (Qiagen, Valencia, CA, USA) from 3 to 5 ml of plasma.

Free-circulating nucleic acids in biological fluids are usually bound to proteins or enveloped in vesicles, requiring an efficient lysis step in order to release cfDNA. Therefore, the first step for cfDNA extraction (Fig. 15) had to be a lysis process. Samples were lysed under highly denaturing conditions at elevated temperatures (60°C) in the presence of proteinase K and Buffer ACL, which together ensure the inactivation of plasma DNases and RNases and the complete release of nucleic acids from bound proteins, lipids, and vesicles[192].

In the second step, the lysate was transferred onto a QIAamp Mini column. cfDNA was adsorbed from the relatively large volume of lysate onto the small silica membrane of the QIAmp Mini column as the lysate was drawn through by the pressure applied by a vacuum pump (Fig.15). Binding conditions were adjusted by adding Buffer ACB to allow optimal binding of the cfDNA to the silica membrane; salt and pH conditions ensured that proteins and other contaminants were not retained on the QIAamp Mini membrane[192].

The third step was the removal of residual contaminants. cfDNA remained bound to the membrane, while contaminants were efficiently washed away during 3 wash steps (Fig.15)[192].

In the fourth and final step, elution of highly pure cfDNA was performed using Buffer AVE (Fig. 15)[192]. cfDNA was eluted in 50 µl of the elution buffer.

3.2.3. Measurement of circulating tumour DNA

The investigational part of this study included the detection of the mutations in *KRAS* codon 12 and 13 in cfDNA. The quantification of ctDNA was conducted by Droplet Digital™ PCR (ddPCR) using the ddPCR Mutation Assay (BioRad®, Hercules, CA, USA). The analysis was performed with a ddPCR *KRAS* Multiplex assay, and the results were validated using the single mutation assays for the most common variants G12D, G12V, G12R, G13D.

ddPCR has been developed to provide high-precision, absolute quantification of DNA: ddPCR can measure the number of copies of a target sequence per volume unit by counting the DNA molecules contained in discrete, volumetrically defined water-in-oil droplet partitions[193]. This technique has unparalleled sensitivity, achieved by means of the signal-to-noise increase provided by the use of droplets which, theoretically, encapsulate no more than one molecule of target DNA (limiting dilution).

The first step of the procedure is the preparation of a ddPCR reaction mix, in a way that is entirely similar to any other real-time PCR: the sample of cfDNA previously obtained is combined with the ddPCR supermix, primers, DNA polymerase, and probes labelled with a FAM or HEX reporter fluorophore.

Subsequently, samples are placed into a droplet generator, used to partition each sample into approximately 20,000 nanoliter-sized droplets uniform in volume, thus enabling precise target quantification. Water droplets are suspended in oil. During the partitioning process, background and target DNA (in our case, total cfDNA and fragments containing the *KRAS* gene) are distributed randomly into the droplets. This means that a droplet may contain no nucleic acid template, background DNA sequences, one target DNA sequence or a combination of the previous options (Fig. 16)[193].

A PCR amplification follows; the defining feature of ddPCR is that thanks to the previous partitioning the reaction goes on independently in every single droplet. The amplification protocol was standardised for all mutations to the following conditions: 95°C × 10 min, 94°C × 30 s and 55°C × 60 s (35 cycles), 98°C × 10 min, and 4°C hold.

After the PCR reaction, a droplet reader is employed for fluorescence signal quantification. Droplets are analysed singularly using a two-colour detection system set to detect the fluorophores, FAM and HEX[193]. Positive droplets, containing at least one target DNA molecule, exhibit increased fluorescence compared to negative ones (Fig. 17A). In our reaction, FAM was associated to wild-type *KRAS*, and HEX to mutant *KRAS*.

Data can be viewed in a 2-D plot in which FAM fluorescence is represented on the x-axis and HEX fluorescence on the y-axis[193]. Droplets cluster into four groups: FAM-negative, HEX-negative (double-negative droplets which did not contain a target DNA template), FAM-positive, HEX-negative (droplets containing wild-type *KRAS*), FAM-negative, HEX-positive (droplets containing mutant *KRAS* ctDNA), FAM-positive, HEX-positive (double-positive droplets containing both mutant and wild-type templates) (Fig. 17B).

The QuantaSoft™ software measures the number of positive and negative droplets for both fluorophores (FAM/HEX). The proportion of positive droplets is then fitted to a Poisson distribution; this allows to quantify the number of copies of the target molecule, as copies/ml and fraction of the total cfDNA initially present in the sample[193].

A fluorescence intensity threshold of 3000 was set as cut-off value and all droplets above this threshold were considered as positive for mutant *KRAS*. Samples were deemed *KRAS*-positive when at least 3 HEX-positive droplets above the threshold level were identified.

3.3. Results

3.3.1. Clinical features of the study population

Out of the 24 patients recruited, 14 were males and 10 females. Median age was 67 years old (range: 48-75). The site of the primary tumour was the head or uncinate process of the pancreas in 12 patients and the body or tail of the pancreas in the remaining 12 subjects. Three patients were classifiable as having stage III disease (LAPC), and 21 had stage IV disease (mPDAC) at accrual. In the cases in which a previous resection had been performed and pathologic staging determined, grading was G2 for 3 patients and G3 for 4 patients.

Eight patients received first-line FOLFIRINOX, 15 gemcitabine plus nab-paclitaxel, and one patient was administered a combination of nab-paclitaxel plus 5-FU/LV and oxaliplatin, (NAB-FOLFOX), as part of a phase II trial investigating the activity of this new regimen[194]. 18 patients had an ECOG Performance Status of 0 on the administration of the first cycle of chemotherapy, while 6 were classifiable as having an ECOG PS of 1. The median number of chemotherapy cycles administered was 5.5 (range 1-10).

Best response was assessed by RECIST 1.1 criteria. The best response during follow-up was radiological partial response for 8 patients and stable disease for other 8 patients; unfortunately, 8 patients showed progression of disease at follow-up imaging.

Median PFS was 5.9 months (range 1.8-9.0 months). 12 patients experienced disease progression, and 8 went on to receive second-line chemotherapy. The regimens administered for second-line treatment were FOLFIRINOX (1 patient), gemcitabine plus nab-paclitaxel (1 patient), FOLFIRI (5-FU/LV plus irinotecan, 4 patients), nab-paclitaxel monotherapy (2 patients). One out of the 3 patients with LAPC received 9 cycles of FOLFIRINOX, experienced a partial response, and has been deemed to be a candidate for resection by a multidisciplinary team.

Median OS was not reached, because only 2 patients passed away during follow-up. Median follow-up was 6.5 months (range 1.0-10.0 months).

14 patients had liver metastases, 5 patients had peritoneal deposits, 4 had lung nodules, 1 had bone lesions, 18 did not undergo resection of the primary tumour, and 1 had a local relapse.

Nine patients were deemed to have a subjectively high tumour burden at baseline; 2 patients were found to have an objectively low tumour load, 13 a moderate tumour load and 9 a high tumour load at baseline.

CA19.9 average concentration at baseline was 4140.8 U/ml (median 1038.7 U/ml, range 0.6-20450.0 U/ml); at best response, its average was 2955.7 U/ml (median 975.2 U/ml, range 2.0-18772.0 U/ml). CA19.9 average levels stratified by response to treatment can be found in Table 8.

3.3.2. Circulating tumour DNA interrogation

Mutant *KRAS* was detected in 22 patients throughout the entire follow-up of the 24 patients recruited, indicating the presence of ctDNA. The most common mutation was G12D, found in 14 patients (58%), followed by G12V, detected in 4 patients (17%), and G12R and G13D, both present in 2 patients (8% each). Two patients had plasma samples persistently negative for mutant *KRAS* (Table 8).

19 patients had detectable somatic mutations in *KRAS* at baseline (79%). In *KRAS*-positive samples, the average baseline amount of ctDNA was 5030 copies/ml (median 260 copies/ml, range 80-64800 copies/ml). On average, *KRAS*-mutant ctDNA accounted for 5.41% of total cfDNA (median 1.60%, range 0.08-38.60%). 22 patients had a second plasma sample available for analysis. After the first cycle of chemotherapy, *KRAS* somatic mutations could not be detected in 8 patients (36%), while 14 patients (63%) had measurable levels of mutant *KRAS*. The average concentration of ctDNA in the 14 *KRAS*-positive patients was 1280 copies/ml (median 240, range 100-10900 copies/ml); ctDNA made up for an average percentage of 2.73% of total cfDNA (median 1.35%, range 0.09-14.00%). 14 patients provided a third blood sample, and 4 a fourth one (Table 9).

3.3.3. Monitoring of circulating tumour DNA levels during chemotherapy

Longitudinal monitoring of the levels of *KRAS* somatic mutations in plasma was possible for 22 out of the 24 patients enrolled (91.7%). The remaining two patients were lost to follow-up due to disease-related complications. Other two patients provided two samples in which no ctDNA could be detected. Among the patients with multiple follow-up samples collected, many had ctDNA levels which varied considerably during the course of chemotherapy, thus possibly allowing the use of ctDNA as a biomarker for the assessment of treatment response and early detection of disease progression.

For the majority of patients, ctDNA changes corresponded to radiological follow-up data. Out of 11 *KRAS*-positive patients who developed progressive disease, 8 had an upward trend in ctDNA levels; among the 9 patients who did not experience progression, 6 had a downward trend, with undetectable mutant *KRAS* in the last follow-up sample in 4 patients. However, 3 patients with declining mutant *KRAS* concentrations experienced progression, while 3 patients with no progression at follow-up imaging had rising levels of ctDNA (Fig. 18).

For instance, patient 7, a 64-year-old lady who had previously undergone surgical resection (pT3N0) and had developed recurrent disease in the form of liver metastases, received 5 cycles of first-line FOLFIRINOX but rapidly progressed (CT scan performed at 9 weeks of follow-up). This patient did not have elevated CA19.9 values. The trend for *KRAS* trend in this subject is shown in Fig. 19. After the first cycle, *KRAS* concentration very slightly declined from 1800 to 1700 copies/ml, but after three months peaked at 11100 copies/ml; figures concerning the ctDNA to total cfDNA ratio showed a continuous upward trend from 3.5 to 3.8 and 9.9%.

On the other hand, patient 16, a 69-year-old gentleman with unresected primary tumour and liver metastases, received 9 cycles of FOLFIRINOX and experienced a partial response, confirmed radiologically after 9 weeks of follow-up. CA19.9 concentrations were 5592 U/ml and 4810 U/ml at baseline and at response respectively. ctDNA concentration dropped from 11700 copies/ml (19.4% of total cfDNA) at baseline to 900 copies/ml (3.4% of total cfDNA) after the first cycle,

and further fell to 130 copies/ml (0.13%) after 3 months. The last follow-up blood sample, collected after 4 months of follow-up, showed undetectable mutant *KRAS* (Fig. 20).

The subgroup of patients with no progression of disease and a concordantly downward trend in ctDNA levels had substantially lower levels of ctDNA after the first cycle of chemotherapy; on average, 260 copies/ml vs. 3060 copies/ml at baseline (-91.6%), or as regards ctDNA to total cfDNA ratio, 0.72% vs. 5.20% (-86,1%). This marked fall anticipated the radiological response by an average of 54 days.

ctDNA and CA19.9 trends were also analysed stratifying by response to treatment (Table 10). Patients with disease control had, on average, considerably lower *KRAS* levels at response (610 copies/ml at radiological response vs. 1750 copies/ml at baseline for partial response and 160 copies/ml at radiological response vs. 860 copies/ml at baseline for stable disease respectively). This was true for CA19.9 as well (1080 vs. 2170 U/ml and 1050 vs. 3060 U/ml respectively). Instead, considering all patients who experienced disease progression, *KRAS* levels were almost four-fold increased at progression as compared to baseline (3950 copies/ml at radiological progression vs. 1110 copies/ml at baseline), while CA19.9 concentrations were slightly lower (6500 vs. 7820 U/ml).

However, a comparison between the values of baseline ctDNA stratified by best response did not achieve statistical significance (Kruskal-Wallis test with Dunn's test for multiple comparisons, all p values > 0.17). Similarly, baseline values did not show a statistical difference between patients who encountered disease progression and patients who did not (Mann-Whitney's test, $p = 0.17$).

On the first follow-up sample (15 days after chemotherapy initiation), ctDNA levels for patients with disease control at the end of the follow-up and patients who experienced disease progression were not statistically different (Mann-Whitney's test, $p = 0.24$). For the sample collected two months after the start of treatment, statistical analysis identified a marginal trend towards significance for the same comparison (Mann-Whitney's test, $p = 0.10$).

We analysed ctDNA level trends for patients with and without progression of disease by using

a ctDNA change index, defined as $([ctDNA]_t - [ctDNA]_{t=0})/[ctDNA]_{t=0}$, where t = 0 refers to baseline. Statistical analysis did not reveal a significant difference between progressing and not progressing patients as regards the trend indexes relative both to the sample collected 15 days after chemotherapy initiation (p = 0.12) and to the sample drawn 60 days after chemotherapy institution (p = 0.14). Patients with disease control had an average ctDNA change of -48.8% at 15 days of treatment, while patients who encountered progression had an average ctDNA change of +250.0% at the same time point (Fig. 21).

We distinguished two groups of patients according to a cut-off of 6 months as regards PFS; patients who enjoyed a longer progression-free survival had an average ctDNA change of -33.3% after the first chemotherapy cycle, while patients who had shorter PFS had a ctDNA change index of +1754%. However, the difference was not statistically significant (p = 0.45) (Fig. 22).

3.3.4. Correlation and survival analyses

A correlation analysis between baseline mutant *KRAS* and CA19.9 levels and various clinical variables was carried out. None of the correlations considering ctDNA levels achieved statistical significance, but three of them showed a distinct trend towards significance. Baseline mutant *KRAS* concentrations were moderately correlated with the baseline tumour burden, calculated as the total of the diameters of RECIST 1.1 target lesions (Pearson's r = 0.39, p = 0.06), with progression-free survival (Pearson's r = -0.36, p = 0.08), and overall survival (Pearson's r = -0.37, p = 0.06). CA19.9 levels at baseline were moderately correlated with tumour load as well (Pearson's r = 0.37, p = 0.07).

Survival analysis did not show any statistical difference between patients who were *KRAS*-positive and *KRAS*-negative at baseline as regards progression-free survival (log-rank, Mantel-Cox test, p = 0.14) and overall survival (log-rank, Mantel-Cox test, p = 0.84). However, it is worth noting how only 1 patient out of the 5 who were *KRAS*-negative at baseline experienced progression of disease and 4 are still progression-free, while 11 patients out of 19 with detectable ctDNA at baseline have progressed up to the present time (Fig. 23).

We also examined whether there was any difference in PFS between the groups of patients who were *KRAS*-positive and *KRAS*-negative at 15 days of treatment; statistical analysis did not confirm the presence of any (log-rank test, Mantel-Cox test, p = 0.19) (Fig. 24).

Finally, we chose to investigate whether patients who had stable or declining ctDNA after 15 days of treatment enjoyed a longer PFS than patients who showed an upward trend. The difference was statistically significant (log-rank, Mantel-Cox test p = 0.037, hazard ratio 0.05, 95% CI 0.01-0.83) (Fig. 25).

Post-hoc power analysis showed that the statistical power for this sample is <0.8.

3.4. Discussion

Our work is, to the best of the author's knowledge, the largest one investigating the possible use of ctDNA as a marker of response to treatment in advanced pancreatic cancer. When the recruitment started, there were no studies targeting this issue in the literature. Very recently, a paper which extensively deals with the chance to monitor chemotherapy efficacy with the liquid biopsy has been published by a Norwegian research group, Tjensvoll and colleagues from Stavanger University Hospital[189]. This study, however, was conducted on an even smaller number of subjects than our own.

Our population sample was composed of slightly younger patients than the average for patients diagnosed with PDAC (67 vs. 71 years old), possibly because we selected subjects who were fit for the newer and more aggressive chemotherapy regimens. Half of our patients had a primary tumour in the body or tail of the pancreas, compared to the 20% reported in the literature.

The length of the follow-up was higher than that of Tjensvoll et al. (6.5 vs. 3.7 months). Our patients seemed to enjoy a relatively better prognosis. In fact, progression was encountered in 12 patients out of 24 (50%), as opposed to the 11 out of 14 (79%) described by Tjensvoll and co-workers[189]. Similarly, only 2 patients deceased during the follow-up (8%), compared to 10 (71%) in the Norwegian study. The underlying reason may be that, in the Norwegian cohort, about two-

fifths (6/14, 42%) of patients received gemcitabine monotherapy, whereas all our patients benefited from more active regimens. As a result, median OS data were not available for our cohort.

Considering all the samples collected, we reported a higher proportion of *KRAS*-positive patients than any other previous study (91.7%). This may be due to several factors: most studies to date only determined *KRAS* mutational status only once; we employed ddPCR, one of the most sensitive methodologies available at the present time; we only recruited patients with LAPC and mPDAC; we did not analyse a group of healthy controls to set a cut-off value for *KRAS* mutations positivity, but used the arbitrary threshold of 3000 as for fluorescence intensity. It would be very interesting to analyse tumour biopsies from these patients and to confirm the high proportion of mutations in *KRAS* with the current gold-standard methodology of tumour tissue molecular analysis.

We mentioned that, however, only 19 patients were *KRAS*-positive at baseline. Three subjects had undetectable mutant *KRAS* in plasma at chemotherapy institution, but later follow-up plasma samples revealed measurable quantities of ctDNA. This might be explained by the emergence of progressive disease during follow-up, but only one out of the 3 patients in question encountered progression. As the methodology employed, ddPCR, has a sensitivity higher than 99.9%, it is unlikely that low concentrations of ctDNA at baseline went missed. However, the technique is quite complex, and pre-analytical errors might have occurred. Molecular analysis of tumour tissue could prove instrumental in these cases to clarify the mutational status of *KRAS*. The issue of patients who are *KRAS*-negative at baseline but are identified as *KRAS*-positive during follow-up needs to be discussed in the future when more evidence about the mechanisms of ctDNA shedding is available.

The case of patient 7 clearly shows how urgently a new tumour marker for monitoring of treatment response is needed, because this patient did not have elevated CA19.9 levels despite the advanced stage of the disease. At least 10% of Caucasian patients remain CA19.9-negative in this condition because of a genetic defect in the enzyme producing the polysaccharidic antigen in

question[50].

The borderline significant correlation (p = 0.06) highlighted for baseline *KRAS* levels and tumour burden supports our hypothesis that the amount of ctDNA in the blood is related to tumour load. Nonetheless, the range of *KRAS* levels seen at baseline was extremely high: 90 to 64800 copies/ml, or 0.08 to 38.6% of total cfDNA. The mechanism through which pancreatic neoplasms release higher or lower quantities of tumour DNA in the bloodstream needs to be further elucidated.

The ranges for patients who responded and did not respond to chemotherapy were largely overlapping; many patients who developed progression of disease started out with values as low as 120 copies/ml, and in one case even with undetectable ctDNA. Intriguingly, however, the subject who had the highest overall concentration of ctDNA (64800 copies/ml, 38.6% of total cfDNA), patient 21, provided only one sample because she received one cycle of gemcitabine plus nab-paclitaxel before being lost to follow-up because of disease-related complications.

We could speculate that ctDNA concentration trends may be more informative than *KRAS* baseline levels. Although statistical significance was not achieved because of our small sample size, in the group of patients with disease control ctDNA levels after one cycle of chemotherapy were almost halved as compared to baseline, while in the group of patients progressing the same figure was increased on average 2.5-fold over the same period.

In our cohort, we were not able to prove that dynamic changes in ctDNA levels allow to anticipate the radiological establishment of progression. The reason for this probably lies with the fact that, in our study, blood samples were not drawn at regular time intervals, but, potentially, there could be quite a long time span between the sample collected after three months of treatment and the one at progression. One may hypothesise that a liquid biopsy performed some weeks (or even months) before progression may reveal increasing levels of mutant *KRAS*, thus allowing the clinician to predict it. However, Tjensvoll et al. pointed out that an increase in ctDNA levels could only be observed at the same time as imaging confirmed progression in some patients[189].

Instead, considering a subgroup of patients (n = 6) who responded to chemotherapy and had, consistently with the hypothesis that mutant *KRAS* may be used as a surrogate biomarker for tumour load, a downward trend in ctDNA levels, a considerable decline in the values was evident already on the second liquid biopsy, 15 days after chemotherapy institution. This means that the decrease in ctDNA values preceded the radiological confirmation of response to therapy (partial response/stable disease) by almost two months. Remarkable drops in mutant *KRAS* levels after the first chemotherapy cycle may, therefore, be a predictive marker for a good treatment response. This aspect had never been pointed out in previous works. It should also be noted that patients achieving disease control, on average, showed a decline in ctDNA levels at 15 days of treatment while patients who encountered progression had rising values; however, the comparison between the two groups did not achieve statistical significance.

As regards the 6 patients with divergent ctDNA levels trends and treatment responses, it should be said that the case of patients with increasing values of mutant *KRAS* in the blood and no radiological evidence of progression could be explained by the fact that the median follow-up time in our study was relatively short. A subsequent CT/MRI scan may very possibly unveil progressive disease, anticipated by several months by the upward trend of ctDNA levels. Out of the 3 patient who experienced progression of disease but had declining mutant *KRAS* in consecutive plasma samples, 2 had peritoneal nodules, which may release tumour DNA into the ascitic fluid instead of the bloodstream.

Many studies reported ctDNA positivity as a negative prognostic factor[182,185,189]. In our cohort, *KRAS* positivity at baseline and at 15 days of treatment was not proven to have an adverse impact on PFS and OS. This may be correlated with the low proportion of *KRAS*-negative patients we reported, compared to other studies, thus not allowing the achievement of statistical significance, also given our small sample size and short follow-up. However, Fig. 23 clearly shows that Kaplan-Meier curves for PFS in patients *KRAS*-positive and *KRAS*-negative at baseline do not overlap. Moreover, the correlation analysis identified a trend towards significance for baseline *KRAS* levels

and progression-free survival and baseline *KRAS* and overall survival ($p = 0.08$ and $p = 0.06$ respectively). Thereby, we are hopeful that a longer follow-up and the recruitment of a higher number of participants may unveil a statistically significant difference. The fact that post-hoc power analysis indicated a statistical power inferior to 0.8 confirms that there is still room for improvement and our results may be regarded as preliminary.

The finding that patients who have stable or declining ctDNA after the first cycle of chemotherapy have a significantly longer PFS as opposed to patients who have rising values of ctDNA is very intriguing, because it suggests that the early assessment of ctDNA levels during chemotherapy may identify a subgroup of patients with better prognosis.

The limitations of the current study are the small sample size ($N = 24$), the short follow-up time (median 6.5 months) and the fact that ctDNA levels were not regularly monitored throughout the entire duration of treatment. Thereby, we aim to continue with the follow-up of the patients included and to enrol others so as to at least double our sample. The newly-recruited patients will be drawn a blood sample on a monthly basis, as in the study by Tjensvoll and colleagues.

Chapter 4. Conclusions

The results of our pilot study support the hypothesis that ctDNA may be adopted as a new marker for monitoring treatment efficacy in advanced pancreatic cancer. We confirmed, as shown by some works in the literature, that ctDNA detection in patients with late-stage pancreatic cancer is feasible in the vast majority of cases. Additional findings are that early changes (after 1 cycle of chemotherapy) in ctDNA levels could predict response to treatment and PFS and that *KRAS*-negative patients at baseline may represent a subgroup with more favourable prognosis. However, future trials further investigating this matter under highly rigorous conditions in larger cohorts are warranted.

Despite the substantial advancements achieved in recent years, the prospects of the vast majority of patients newly diagnosed with pancreatic cancer are still grim. However, much research is being carried out at present, and the estimates that predict PDAC to become the second cancer killer in the United States within the next fifteen years[6] likely imply that substantial investments will be made in this field. A breakthrough may be on the horizon, as suggested by an editorial very recently appeared in *The Lancet Oncology* with the intriguing title "Pancreatic cancer: cause for optimism?"[195].

Two new active regimens for advanced disease have been established in the last five years, FOLFIRINOX[102] and gemcitabine plus nab-paclitaxel[103]; the development of new therapies thanks to large randomised clinical trials is, however, imperative. Earlier this year, positive results regarding two new promising adjuvant regimens were presented, gemcitabine plus capecitabine[82] in Europe and the oral fluoropyrimidine S-1[81] in Japan. As regards surgical techniques, laparoscopy for distal pancreatectomy is being investigated as an alternative to conventional open surgery[68,69], which will possibly allow faster recovery from surgery and earlier initiation and better toleration of adjuvant treatment. A number of targeted therapy agents such as mitogen-activated protein kinase inhibitors[196] or VEGF inhibitors[197,198] have been unsuccessful in prolonging patients' survival, but many others are being examined in currently ongoing clinical trials. Pancreatic cancer is generally

regarded as resistant to immunotherapy, but initial evidence from recent studies suggests that this deadly malignancy might be sensitised to immunotherapy[199,200]. Finally, in the near future, the analysis of circulating tumour DNA might play a part in allowing optimal management of patients, thus becoming a useful instrument in the fight against the disease which was described as "the ruthless dictator of all cancers"[1].

The benefits of the analysis of circulating tumour DNA are that it is a highly specific, minimally invasive and relatively inexpensive test. As a consequence, the liquid biopsy is easily repeatable: the chance to longitudinally compare samples drawn from the same patient, thus providing real-time assessment of treatment efficacy or resistance, is another major strength of this approach.

The basis of the high specificity of ctDNA analysis is the fact that the somatic mutations found in circulating DNA derive from the neoplasm, and these mutations are an exquisite feature of malignant cells. As regards sensitivity, research carried out in different cancers have demonstrated that ctDNA is detectable in most patients with advanced disease[176]. Previous works found detectable ctDNA levels in 25-85% of PDAC patients[176,182,183,185]. Our study found an even higher percentage of *KRAS*-positive patients with advanced pancreatic adenocarcinoma.

There are many potential applications of this technique, such as monitoring of tumour load and treatment response, identification of genetic determinants for therapy, assessment of tumour heterogeneity and resistance to targeted therapy, monitoring of minimal residual disease and early detection of cancer.

Pancreatic adenocarcinoma has been defined as the ideal disease in which liquid biopsy may prove useful[190]. Anatomical and clinical factors make it particularly difficult to obtain tumour tissue specimens; the current serum biomarker, CA19.9, is not sufficiently sensitive and specific. A very limited number of point mutations in a single oncogene, *KRAS*, act as founder genetic alterations in almost all PDACs; as a result, mutant *KRAS* has been the subject of the majority of studies targeting ctDNA in pancreatic cancer. The tumour suppressor genes *CDKN2A*, *TP53*, and *SMAD4* are mutated in more than half of PDACs. Thereby, it has been hypothesised that

employing an expanded panel of somatic mutations to include alterations in these genes alongside with *KRAS* mutations could further increase the proportion of ctDNA-positive patients and provide additional information[189].

However, there are still many open issues to address before ctDNA can undergo clinical implementation. The liquid biopsy needs to be validated as a biomarker in large prospective studies with a statistically powerful sample size in which blood samples are collected on a regular basis throughout treatment; the results obtained will have to be correlated with clinical variables[129]. In these studies, a comparison between ctDNA, conventional markers and imaging should be performed; a ctDNA threshold value for clinical relevance, as for every biomarker, is required.

Another matter is the lack of consensus on which is the most efficient approach for the quantification and analysis of ctDNA. Such methodologies are still in a developmental stage; several steps of the process such as blood collection, processing, storage, DNA extraction and quantification, and target molecules need to be standardised to obtain a high volume of comparable data[129,190].

Quite a lot about the dynamics of ctDNA shedding is still unknown. A number of variables, such as tumour size, site of lesions, and systemic treatment could impact on ctDNA release. The timing of molecular monitoring in connection with therapy, in particular, may play a substantial role. A high amount of ctDNA could be released into the bloodstream following chemotherapy due to massive cancer cell death; a blood sample drawn during or immediately after treatment administration could also reveal various allelic fractions of ctDNA[129].

There is another serious issue to be tackled. Even if an increase in ctDNA levels was proven to be highly predictive of disease progression, the appropriate management of a patient showing such a trend remains to be determined. It may be appropriate to analyse ctDNA after each chemotherapy cycle, trying to determine a cut-off point after which patients should be randomised to switch chemotherapy regimen for "liquid biopsy progression" or carry on with the same regimen until radiological confirmation of progression, and compare survival rates. Nonetheless, there is little

point in proving that a patient is not responding to treatment a greater number of effective treatments for pancreatic cancer are available[187].

Our pilot study, similarly to that by Tjensvoll et al.[189], provides preliminary evidence supporting ctDNA utilisation as a marker of response to systemic treatment and progression in advanced pancreatic cancer. At the moment, there are no available studies in which ctDNA was used to diagnose pancreatic cancer; only a small trial looked into its employment as a tumour marker in the adjuvant setting[183]. Therefore, these potential applications should be the subject of future research.

By way of a conclusion, the liquid biopsy holds great promise, but a significant amount of further data is needed before this methodology can enter routine clinical activity.

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Tables

Table 1. Frequency of signs and symptoms at presentation. Modified from Porta et al., 2005 (Ref. 29, table 2).

Symptoms	**Frequency**
Asthenia	86%
Weight loss	85%
Anorexia	83%
Abdominal pain	79%
Choluria	58%
Nausea	51%
Back pain	49%
Diarrhoea	44%
Vomiting	33%
Pruritus	32%
Steatorrhea	25%
Signs	**Frequency**
Jaundice	55%
Hepatomegaly	30%
Upper abdomen mass	15%
Cachexia	13%
Courvoisier's sign	13%
Ascites	5%
Thrombophlebitis	3%

Table 2. TNM classification for pancreatic cancer according to AJCC VII edition. Modified from Edge and Compton, 2010 (Ref. 54).

Primary tumour (T)

Tx: Primary tumour cannot be assessed

T0: No evidence of primary tumour

T1: Tumour limited to the pancreas, ≤2 cm in greatest dimension

T2: Tumour limited to the pancreas, ≥2 cm in greatest dimension

T3: Tumour extends beyond the pancreas but without involvement of the celiac axis or the superior mesenteric artery

T4: Tumour involves the celiac axis or the superior mesenteric artery (unresectable primary tumour)

Regional lymph nodes (N)

NX: Regional lymph nodes cannot be assessed

N0: No regional lymph node metastasis

N1: Regional lymph node metastasis

Distant metastasis (M)

M0: No distant metastasis

M1: Distant metastasis

Table 3. Staging according to TNM classification, 5-year survival rate, proportion of cases at diagnosis and equivalent stage according to clinical NCCN classification. Modified from Edge and Compton, 2010 (Ref. 54).

Staging	T	N	M	5-year survival	% cases at presentation	Clinical staging
0	Tis	N0	M0	20-25%	10-20%	Resectable
IA	T1	N0	M0			
IB	T2	N0	M0			
IIA	T3	N0	M0			
IIB	T1 T2 T3	N1 N1 N1	M0 M0 M0			
III	T4	Any N	M0	8%	25-35%	Locally advanced (LAPC)
IV	Any T	Any N	M1	2%	45-55%	Metastatic

Table 4. Criteria defining tumour resectability status, according to NCCN Guidelines, version 2.2015. Modified from Conroy et al., 2016 (Ref. 87, table 1).

Resectability status	**Arterial involvement**	**Venous involvement**
Resectable	• No contact with the celiac axis (CA), superior mesenteric artery (SMA) or common hepatic artery (CHA).	• No contact with the superior mesenteric vein (SMV), portal vein (PV) or contact ≤180° without contour irregularity.
Borderline resectable	• Solid tumour in contact with CHA without extension to CA or hepatic artery bifurcation allowing for safe and complete resection and reconstruction. • Solid tumour contact with the CA or SMA ≤180°.	• Solid tumour in contact >180° with the SMV or PV, or in contact ≤180° with contour irregularity of the vein or thrombosis of the vein but with suitable vessels proximal and distal to the site of involvement allowing for safe and complete resection and vein reconstruction. • Solid tumour in contact with the inferior vena cava.
Unresectable	• Solid tumour contact with SMA or CA >180°. • Aortic involvement.	• Unreconstructible SMV/PV due to tumour involvement or occlusion (can be due to tumour or bland thrombus).

Table 5. Comparison of conventional biopsy and liquid biopsy. Modified from McLarty and Yeh, 2015 (Ref. 137, table 1).

Key features	Tissue biopsy	Liquid biopsy
Invasiveness	Yes	Minimal
Sample availability throughout the disease course	No	Yes
Sample stability ex vivo	Stable when processed	Yes
Utility for longitudinal disease monitoring	No	Yes
Cost	High	Low
Processing time	Long (involvement of tissue sectioning, staining and pathologists)	Short
Failure rate	High (quantity not sufficient/tumour not identified)	Low
Selection bias due to intra-tumoral and inter-metastatic heterogeneity	Yes	No

Table 6. Potential clinical applications of liquid biopsy. Modified from Diaz and Bardelli, 2014, and Francis and Stein, 2015 (Ref. 135, table 1; Ref. 136, table 1).

Diagnostic marker	• Early detection • Monitoring of minimal residual disease
Prognostic marker	• Identification of high risk of recurrence • Correlation with changes in tumour load • Estimation of survival
Predictive marker	• Identification of genetic determinants for targeted therapy • Real-time assessment of tumour heterogeneity and development of resistance • Early evaluation of treatment response

Table 7. Clinical features of the study population. ChT, chemotherapy, FOLFIRINOX, 5-fluorouracil, folinic acid, irinotecan, and oxaliplatin, GEM/NAB, gemcitabine plus nab-paclitaxel, NAB-FOLFOX, nab-paclitaxel plus 5-fluorouracil, folinic acid, and oxaliplatin, PS, Performance Status, PR, Partial Response, SD, Stable Disease, PD, Progression of Disease.

Gender	M 14 pts	F 10 pts	
Age	Median 67 y.o.	Range 48-75 y.o.	
Site of primary tumour	Head/uncinate process 12 pts	Body/tail 12 pts	
Staging	Stage III 3 pts	Stage IV 21 pts	
Grading	Pathological staging available in 7 pts	G2 3 pts	G3 4 pts
ChT regimen	FOLFIRINOX 8 pts	GEM/NAB 15 pts	NAB-FOLFOX 1 pt
Performance Status at 1st cycle	PS 0 18 pts	PS 1 6 pts	
Number of ChT cycles administered	Average 5.5	Range 1-10	
Best Response	PR 8 pts	SD 8 pts	PD 8 pts
Progression-Free Survival	Median 5.9 months	Range 1.8-9.0 months	PD 12 pts, 2nd line ChT 8 pts
Overall Survival	Median not reached	Range 1.0-9.9 months	2 pts deceased
Follow-up	Median 6.5 months	Range 1.0-10.0 months	

Table 8. Average CA19.9 levels stratified by treatment response. PR, partial response, SD, stable disease, PD, progression of disease.

Average CA19.9 concentrations	**Baseline [U/ml]**	**Response [U/ml]**
Best response PR group	2165.8	1080.4
Best response SD group	2713.3	1023.5
Best response PD group	7543.2	6503.5
All patients with disease control	2439.5	1045.4
All patients with progression of disease	5823.6	4599.0

Table 9. Results of ctDNA measurements. WT, wild type *KRAS*, meaning mutant *KRAS* undetectable, PD, progression of disease.

Patient		**Baseline sample**	**15 days sample**	**60 days sample**	**PD radiological confirmation sample**	**PD**
1	copies/ml	260	600			YES
	ctDNA/total cfDNA	1.4%	2.3%			
2	copies/ml	WT	160	510		NO
	ctDNA/total cfDNA	WT	0.5%	1.0%		
3	copies/ml	130	3400			YES
	ctDNA/total cfDNA	0.6%	8.6%			
4	copies/ml	220	110	WT	23800	YES
	ctDNA/total cfDNA	1.2%	0.3%	WT	28.4%	
5	copies/ml	WT	WT			NO
	ctDNA/total cfDNA	WT	WT			
6	copies/ml	120	WT	220		YES
	ctDNA/total cfDNA	0.2%	WT	1.1%		
7	copies/ml	1800	1700	11100		YES
	ctDNA/total cfDNA	3.5%	3.8%	9.9%		
8	copies/ml	210	140	WT		NO
	ctDNA/total cfDNA	1.0%	0.4%	WT		
9	copies/ml	WT	10900	600	1200	YES
	ctDNA/total cfDNA	WT	14.0%	0.3%	1.0%	
10	copies/ml	2500	WT			NO
	ctDNA/total cfDNA	3.8%	WT			
11	copies/ml	500	190			NO
	ctDNA/total cfDNA	2.3%	0.2%			

12	copies/ml	120	WT	210		NO
	ctDNA/total cfDNA	0.5%	WT	1.0%		
13	copies/ml	80	WT	WT		NO
	ctDNA/total cfDNA	1.7%	WT	WT		
14	copies/ml	WT	WT			NO
	ctDNA/total cfDNA	WT	WT			
15	copies/ml	50000	420	190	240	YES
	ctDNA/total cfDNA	9.7%	1.4%	1.0%	0.6%	
16	copies/ml	11700	900	130	WT	NO
	ctDNA/total cfDNA	19.4%	3.4%	0.1%	WT	
17	copies/ml	240	245	2700		YES
	ctDNA/total cfDNA	1.6%	1.6%	1.9%		
18	copies/ml	WT	WT	350		NO
	ctDNA/total cfDNA	WT	WT	1.5%		
19	copies/ml	3900	240	170		YES
	ctDNA/total cfDNA	12.5%	1.3%	0.6%		
20	copies/ml	90				NO
	ctDNA/total cfDNA	0.8%				
21	copies/ml	64800				YES
	ctDNA/total cfDNA	38.60%				
22	copies/ml	400	100			YES
	ctDNA/total cfDNA	0.1%	0.1%			
23	copies/ml	3300	310	90		NO
	ctDNA/total cfDNA	3.3%	0.4%	0.8%		
24	copies/ml	150	WT			YES
	ctDNA/total cfDNA	0.6%	WT			

Table 10. Circulating tumour DNA and CA19.9 trends stratified by treatment response. PR, partial response, SD, stable disease, PD, progression of disease.

	Number of patients	***KRAS* at baseline [copies/ml]**	***KRAS* at response [copies/ml]**	**CA19.9 at baseline [U/ml]**	**CA19.9 at response [U/ml]**
Best response PR	8	1750	610	2170	1080
Best response SD	8	860	160	3060	1050
PD	12	1110	3950	7820	6500

Figures

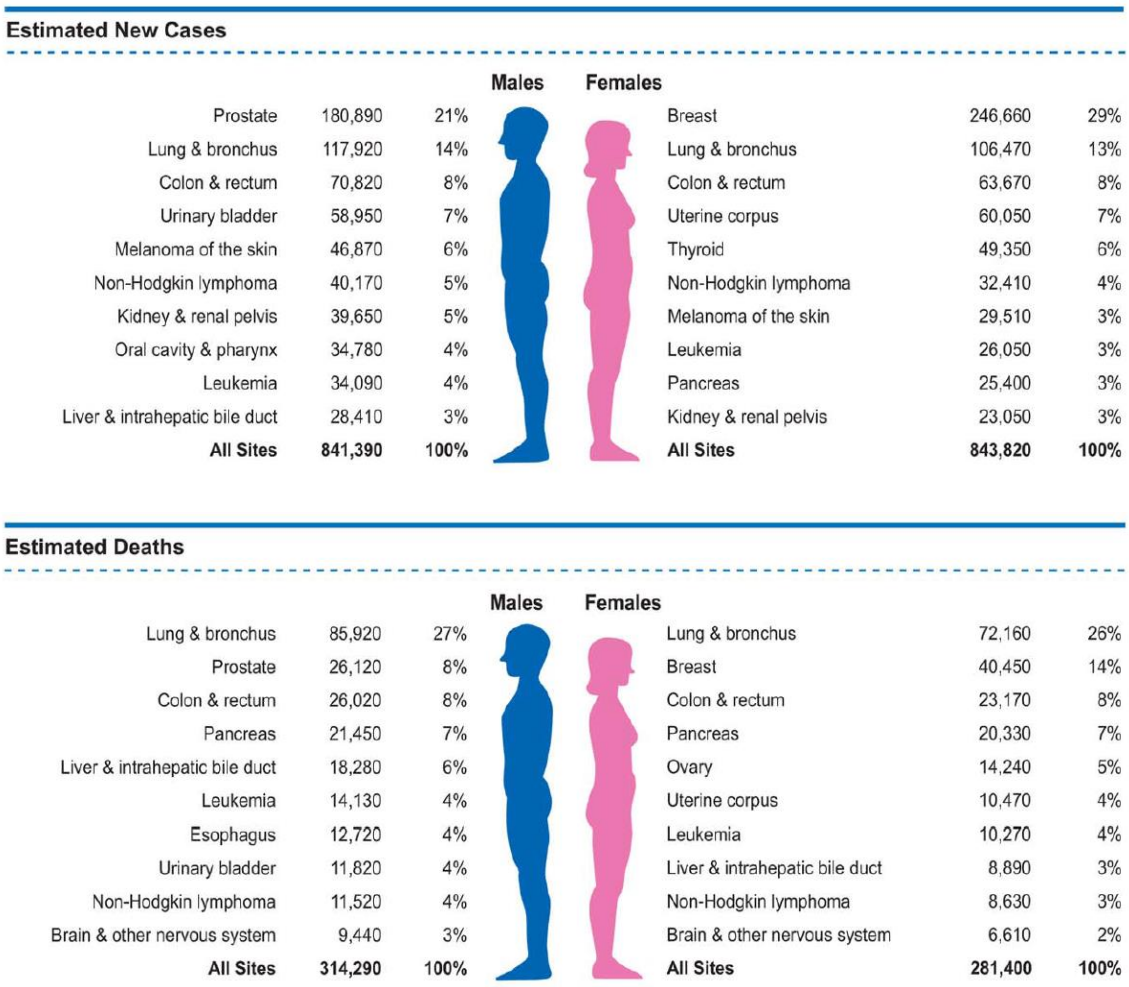


Figure 1. Estimated new cancer diagnoses and deaths in the United States in 2016: the ten leading malignancies. Modified from Siegel et al., 2016 (Ref. 5, fig.1).

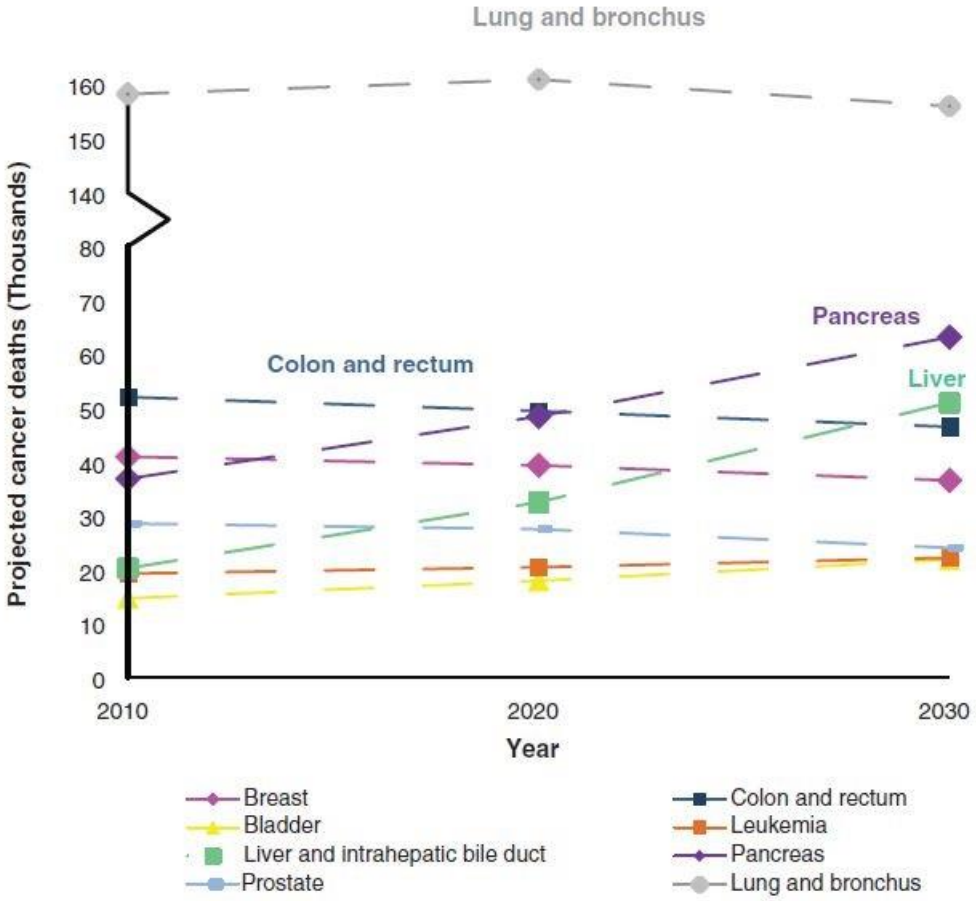


Figure 2. Deaths due to top cancer killers in 2010 and estimates for 2020 and 2030 in the United States: pancreatic cancer will overcome breast and colorectal cancer and will rank second after lung cancer. Modified from Rahib et al., 2014 (Ref. 6, fig.1).

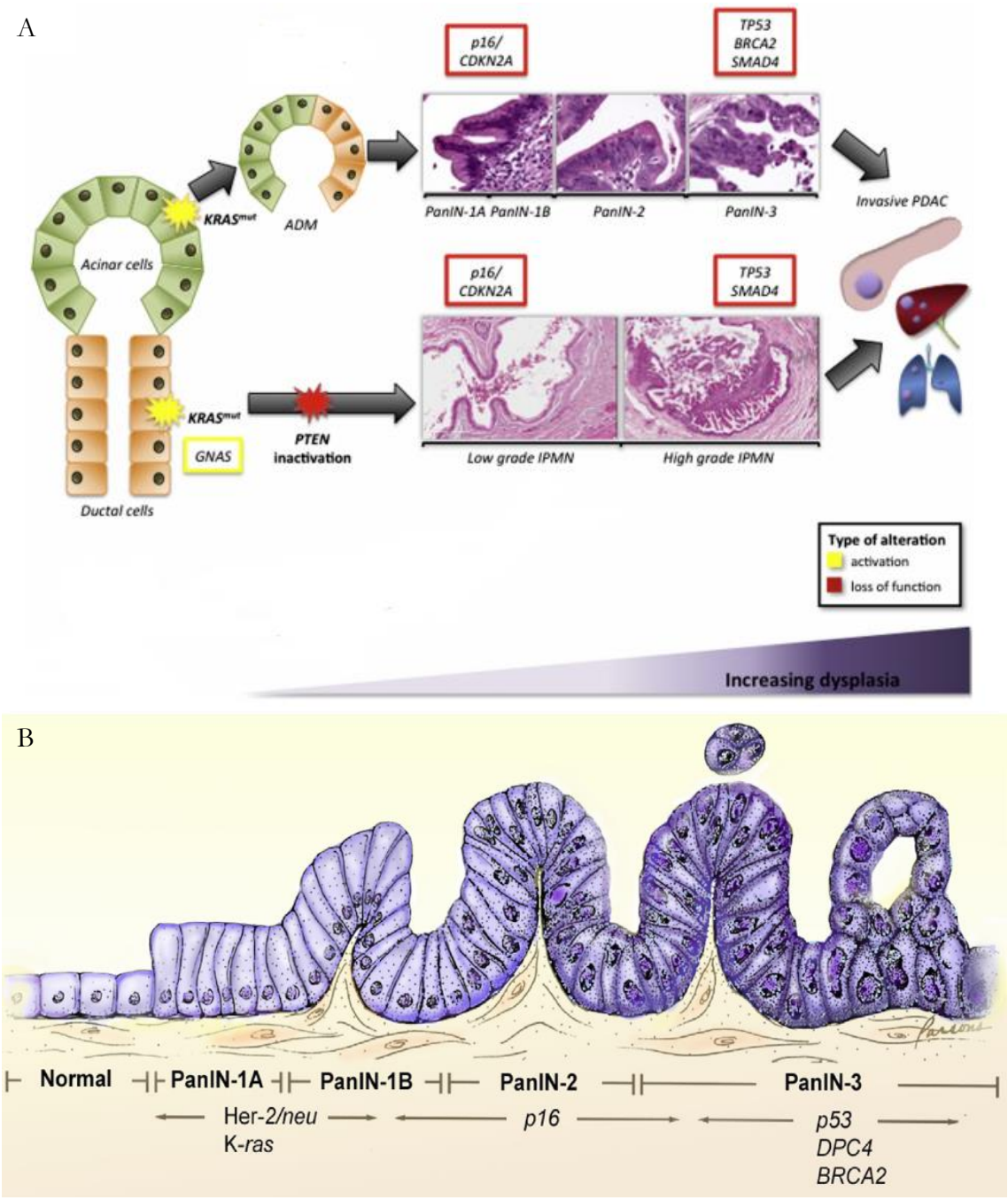


Figure 3. (**A)** Progression model for invasive pancreatic ductal adenocarcinoma (PDAC): acinar-to-ductal metaplasia (ADM), pancreatic intraepithelial neoplasia (PanIN), and intraductal papillary mucinous neoplasia (IPMN). Modified from Neuzillet et al., 2014 (Ref. 13, fig. 1). (**B)** Stages of PanIN and associated mutations in oncogenes and tumour suppressor genes. Modified from Muniraj et al., 2013 (Ref. 4, fig. 13).

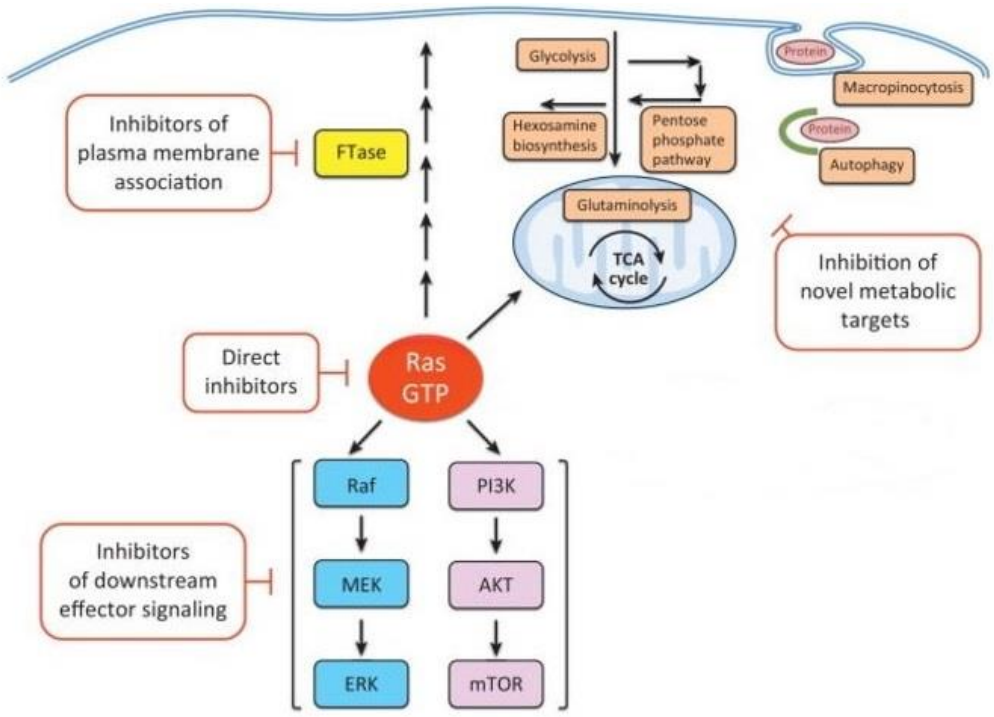


Figure 4. The multiple influences of *KRAS* on the malignant PDAC cell and the possible targets for anti-KRAS inhibitors. Modified from Bryant et al., 2014 (Ref. 20, fig.4).

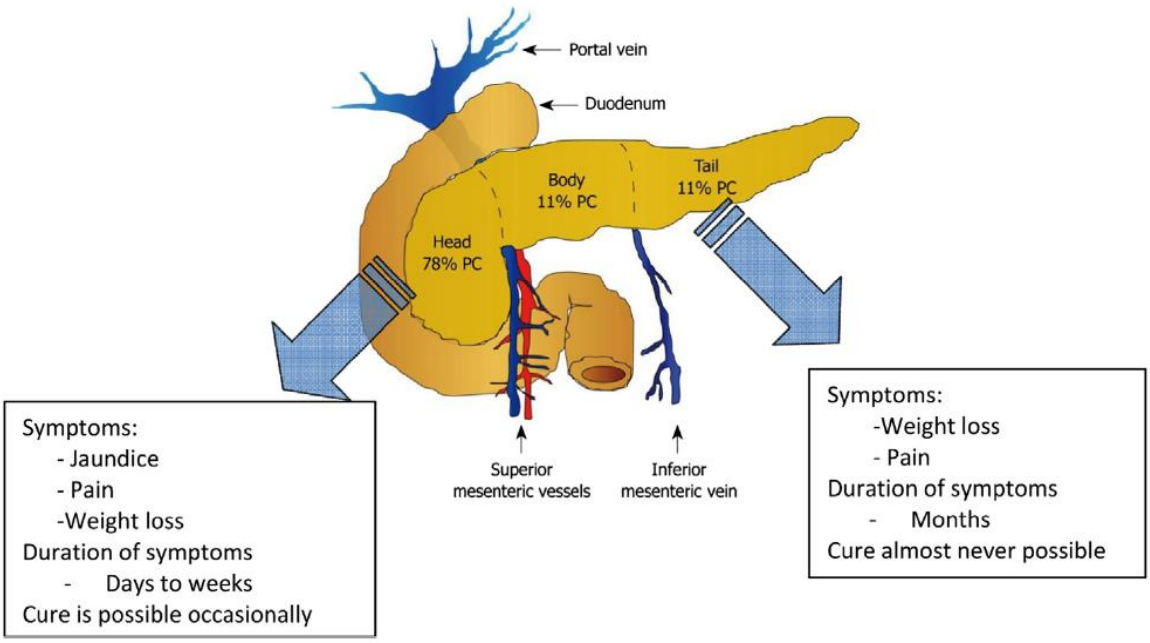


Figure 5. Site of pancreatic cancer and clinical presentation. Modified from Muniraj et al., 2013 (Ref. 4, fig. 16).

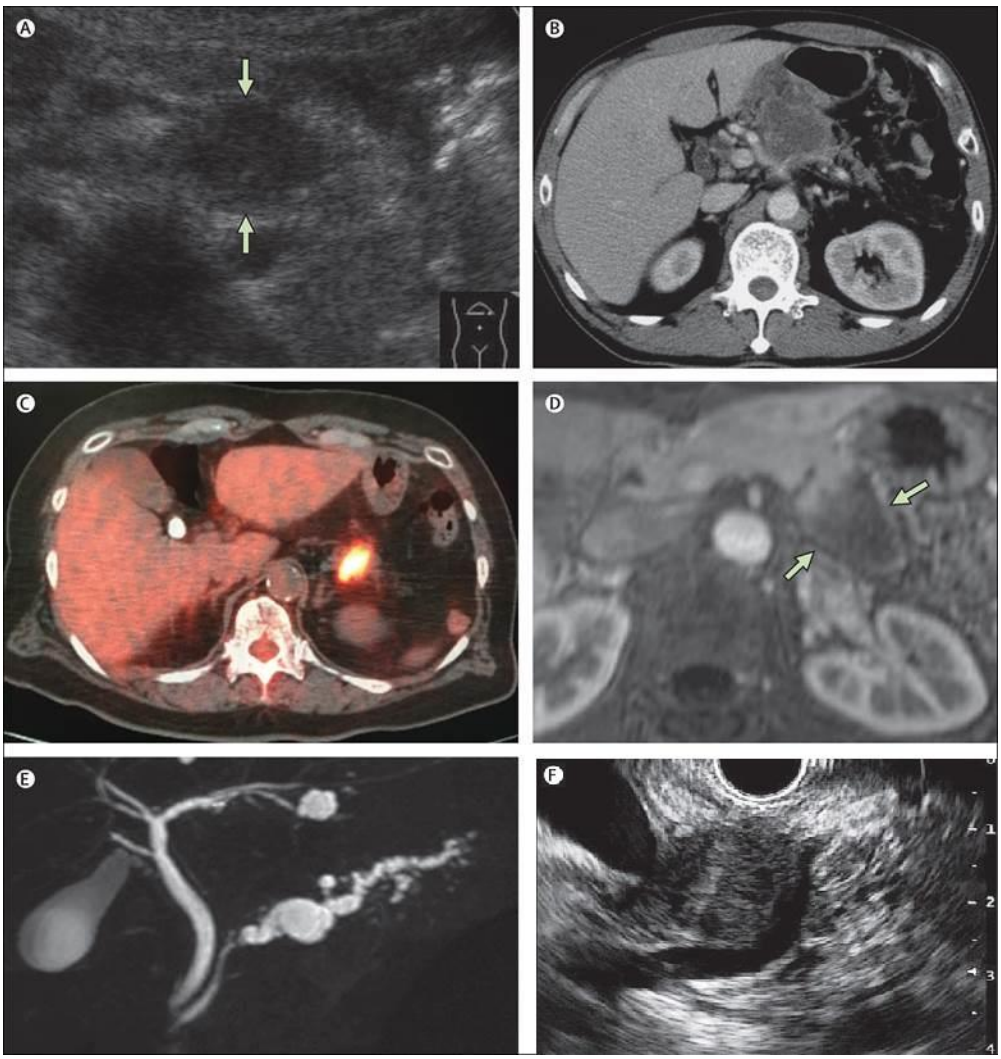


Figure 6. (A) Transabdominal ultrasound (AUS): hypoechoic lesion (arrows) in the head of the pancreas. **(B)** Multidetector-row CT: low-density mass, invading celiac artery and stomach. **(C)** PET-CT: 18FDG uptake in the tail of the pancreas. **(D)** Gadolinium-enhanced MRI: hypointensity mass (arrows) in the tail of the pancreas. **(E)** Magnetic resonance cholangiopancreatography (MRCP): stenosis of the main pancreatic duct with proximal dilation. **(F)** Endoscopic ultrasound (EUS): hypoechoic mass in the body of the pancreas. Modified from Kamisawa et al., 2016 (Ref. 33, fig. 3 and fig. 4).

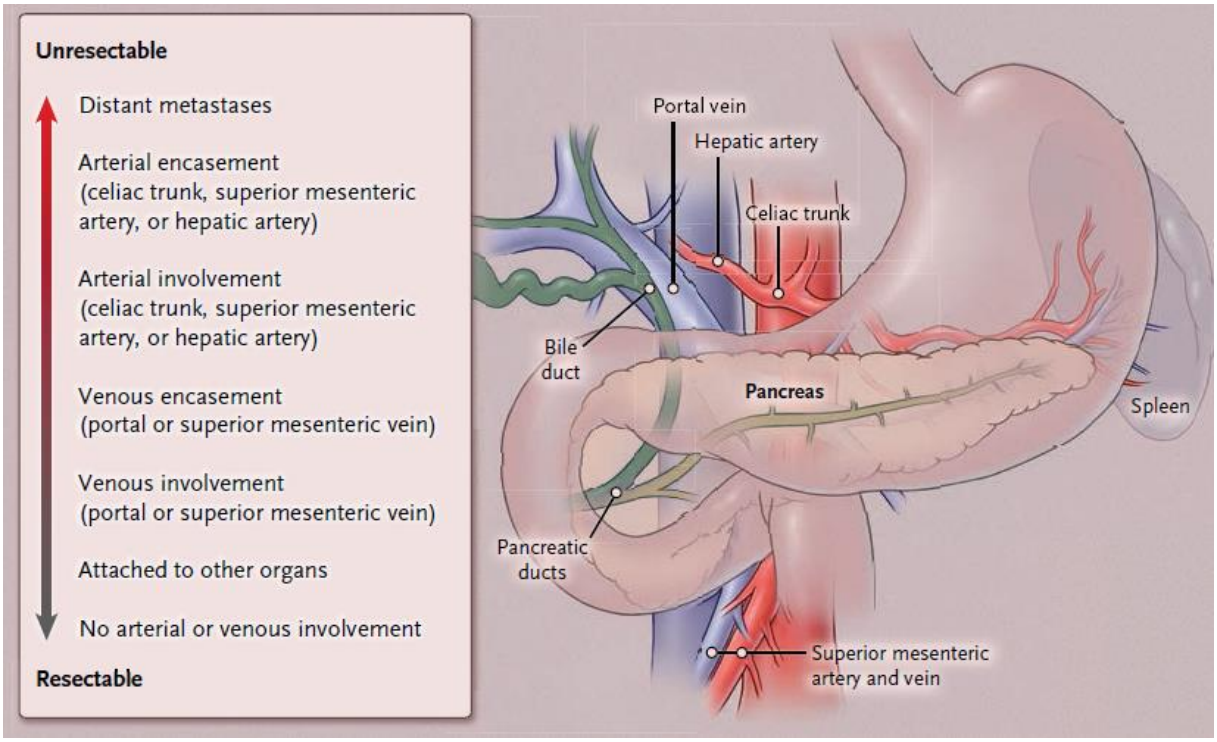


Figure 7. NCCN clinical staging system: pancreatic cancers are classified on a continuum from resectable to unresectable according to the involvement of adjacent structures and the presence of distant metastases. Modified from Ryan et al., 2014 (Ref. 2, fig.3).

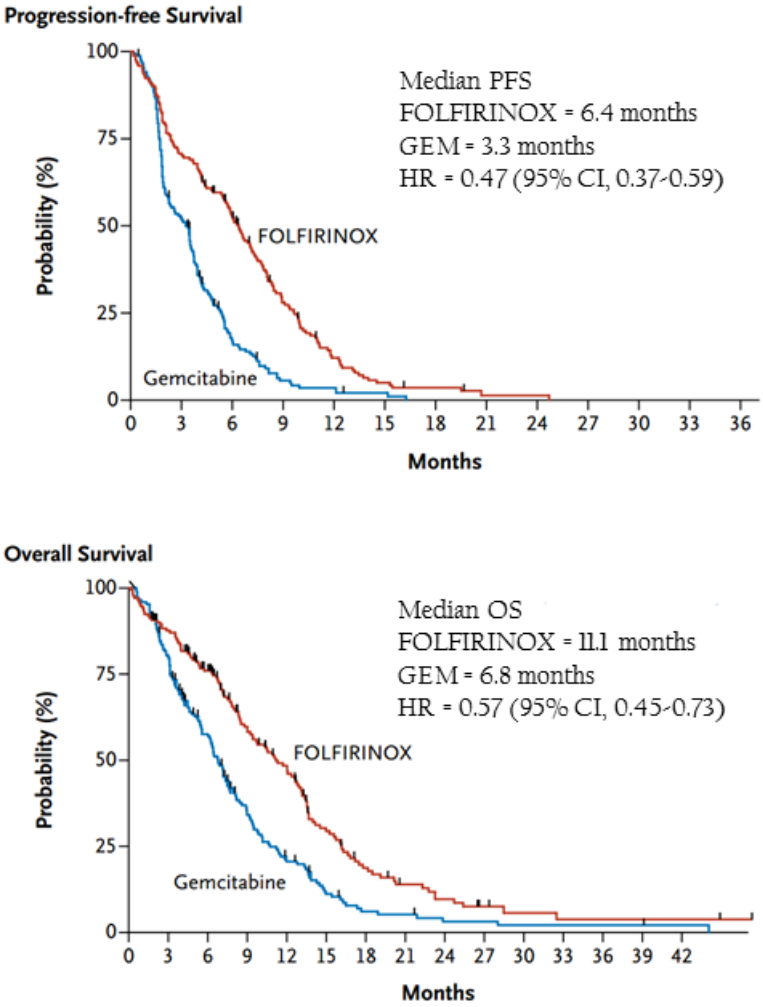


Figure 8. PRODIGE4/ACCORD11 trial: Kaplan-Meier estimates of Progression-Free Survival (PFS) and Overall Survival (OS) for patients with metastatic pancreatic cancer treated with first-line FOLFIRINOX and gemcitabine. Modified from Conroy et al., 2011 (Ref. 102, fig. 1).

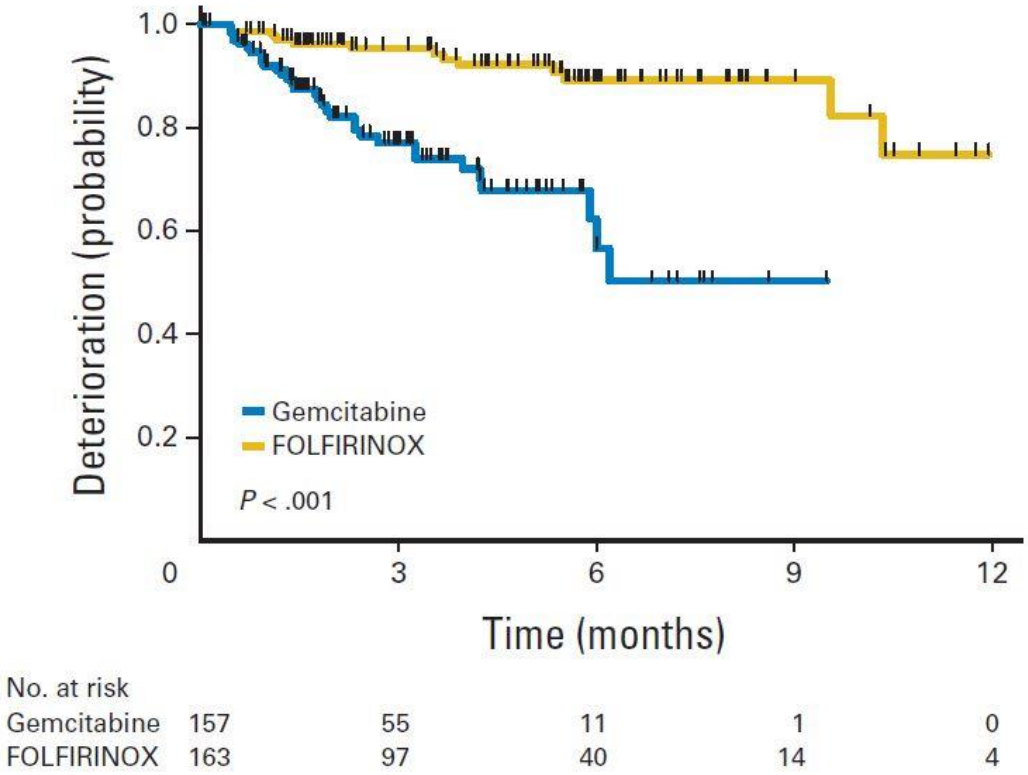


Fig. 9. PRODIGE4/ACCORD11 trial: Kaplan-Meier curves for time until definitive deterioration (decrease of 20 points in the global health status/quality of life score of the EORTC QLQ-C30 questionnaire) for patients with metastatic pancreatic cancer treated with first-line FOLFIRINOX and gemcitabine. Modified from Gourgou-Bourgade, 2013 (Ref. 105, fig.2).

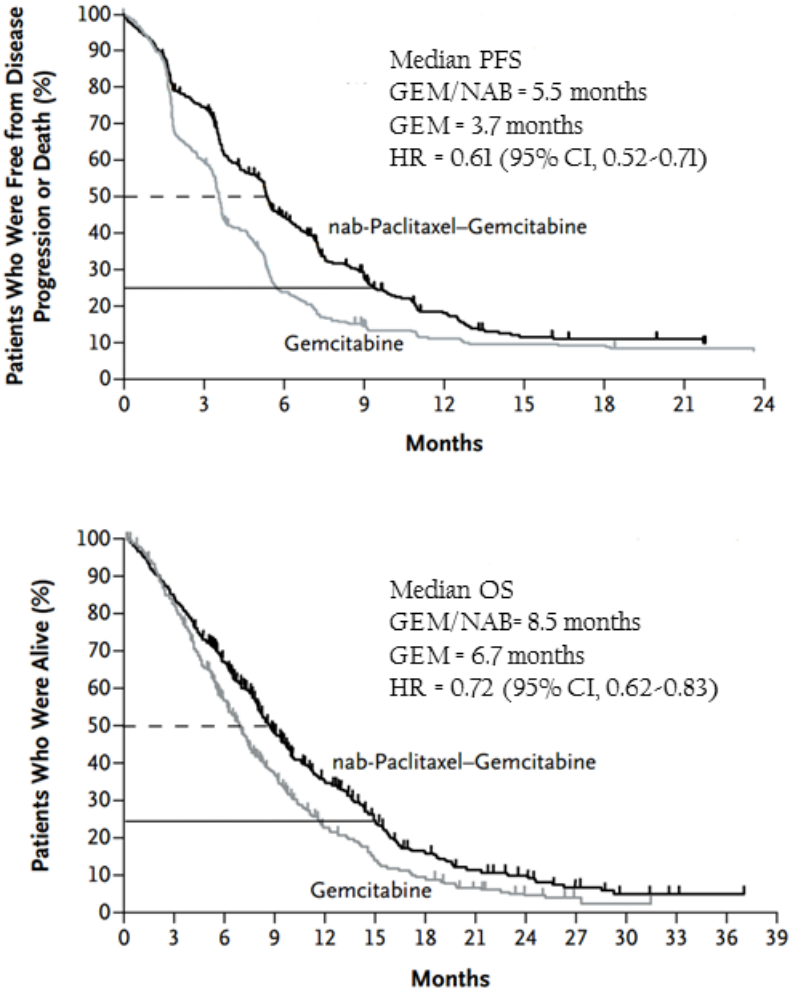


Figure 10. MPACT trial: Kaplan-Meier estimates of Progression-Free Survival (PFS) and Overall Survival (OS) for patients with metastatic pancreatic cancer treated with first-line gemcitabine plus nab-paclitaxel and gemcitabine alone. Modified from von Hoff et al., 2013 (Ref. 103, fig. 1).

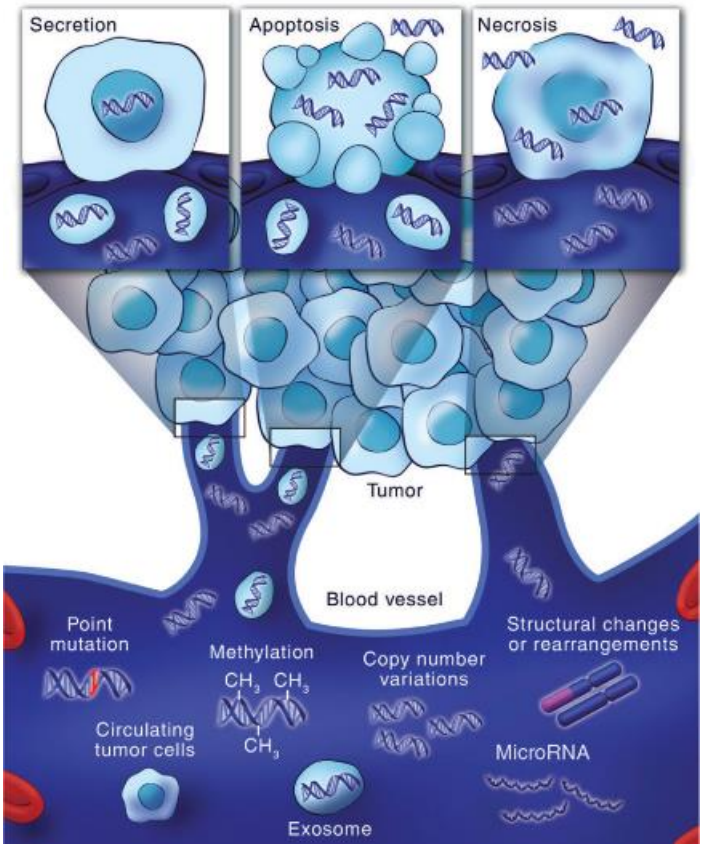


Figure 11. Release of circulating tumour DNA (ctDNA) into the bloodstream. Modified from Diaz and Bardelli, 2014 (Ref. 135, fig. 2).

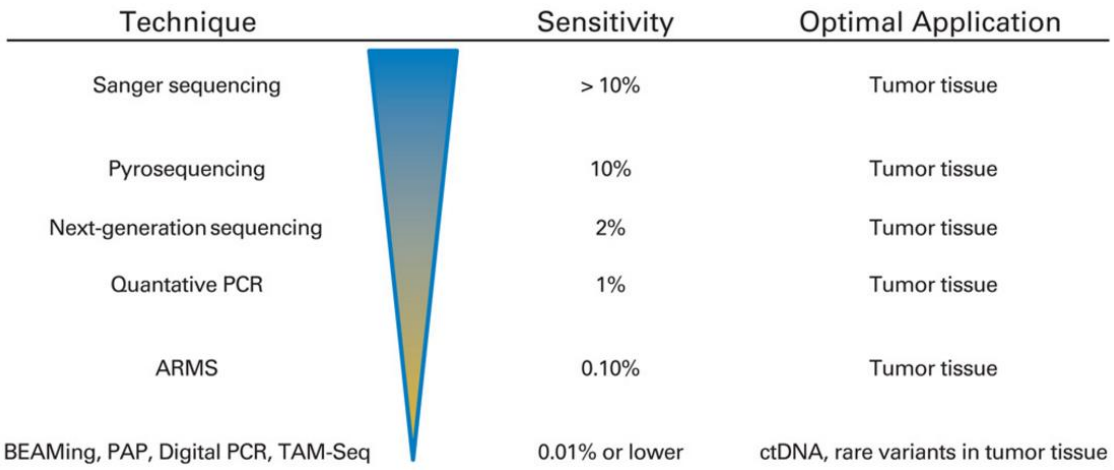


Figure 12. Methodologies for the analysis of tumour DNA, relative sensitivity and optimal application. PCR, polymerase chain reaction; ARMS, amplification refractory mutation system; BEAMing, beads, emulsion, amplification, and magnetics; PAP, pyrophosphorolysis-activated polymerisation; TAM-Seq, tagged-amplicon deep sequencing. Modified from Diaz and Bardelli, 2014 (Ref. 135, fig. 1).

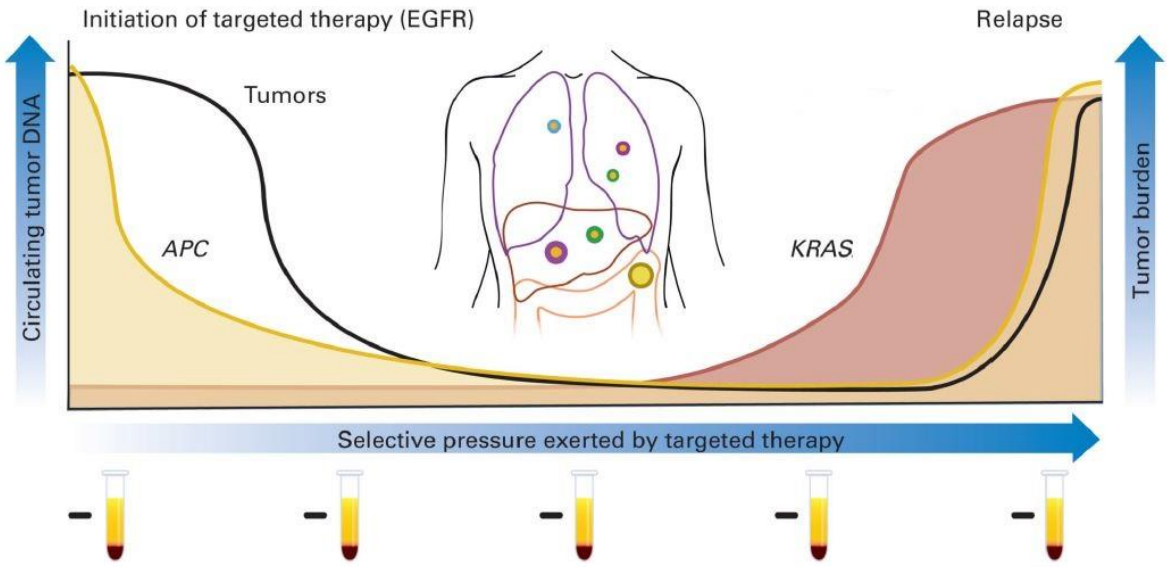


Figure 13. Real-time monitoring of resistance to targeted therapy with liquid biopsy. A patient is diagnosed with colorectal cancer; a tissue biopsy is performed and tumour DNA is analysed. The tumour is found to be *APC* mutant and *KRAS* wild type. At baseline, the liquid biopsy only identifies wild-type *KRAS* fragments. The patient undergoes therapy with an anti–epidermal growth factor receptor (*EGFR*) monoclonal antibody, experiences a clinical response, and has a corresponding decrease in *APC* mutation level in circulating cell-free DNA, further indicating a decline in tumour burden. After some months, dynamic monitoring of circulating tumour DNA (ctDNA) shows detectable *KRAS* mutations, implying the emergence of resistant clones. Progression of disease is only confirmed at a later time point. Test tubes represent samples of plasma on which ctDNA analysis is performed. Modified from Diaz and Bardelli, 2014 (Ref. 135, fig. 3).

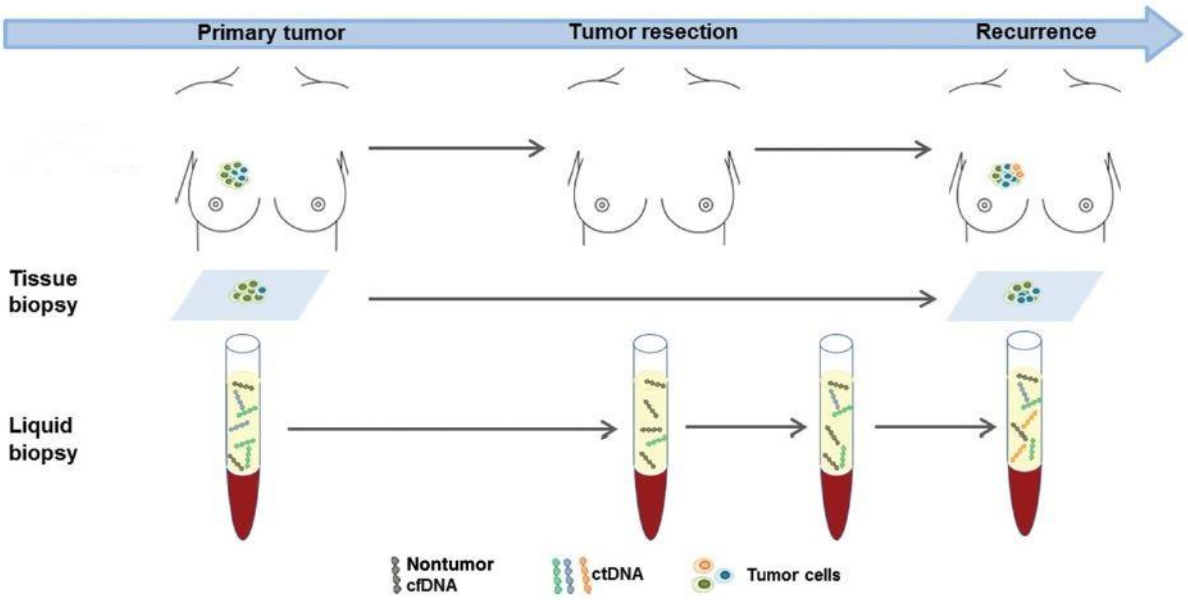


Figure 14. Monitoring of minimal residual disease with liquid biopsy. A patient with breast cancer undergoes curative-intent resection. After surgery, the patient has no evidence of disease; increasing levels of circulating tumour DNA (ctDNA) are detected in subsequent liquid biopsies, possibly long before relapse manifests itself clinically and radiologically. ctDNA analysis after resection could be helpful to identify patients at high-risk of recurrence who can benefit from adjuvant treatment. Modified from Heitzer et al., 2014 (Ref. 130, fig. 2).

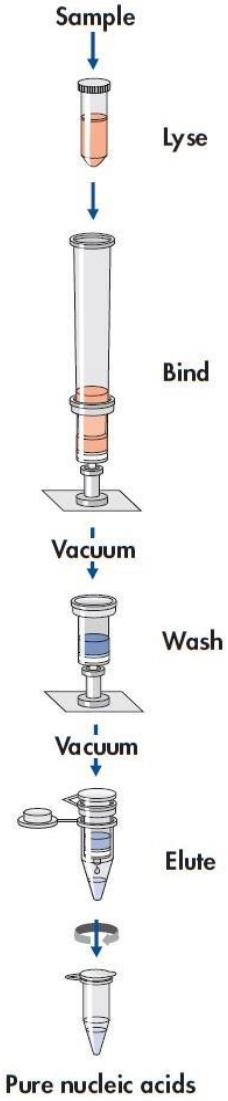


Figure 15. Extraction of circulating cell-free DNA from plasma. Modified from QIAmp® Circulating nucleic acid Kit Handbook, 2011 (Ref. 191, fig. 1).

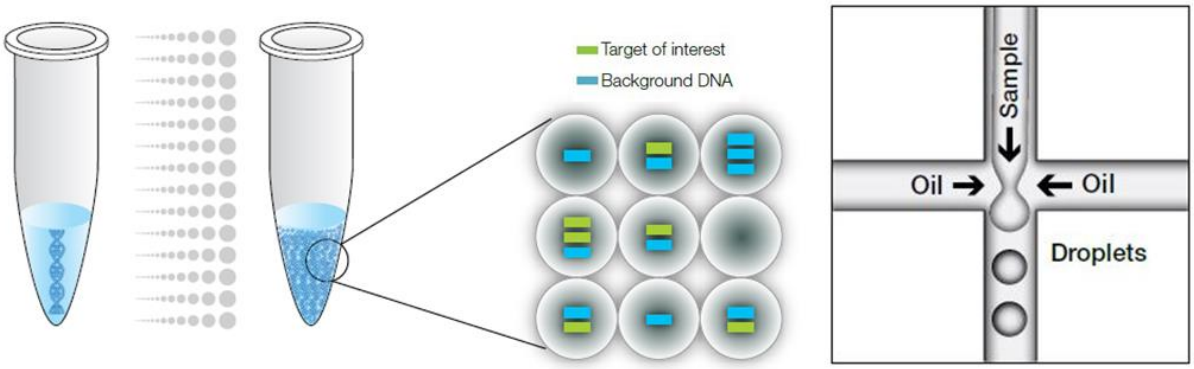


Figure 16. Partitioning in Droplet Digital PCR (ddPCR): a sample is divided into approximately 20,000 droplets all uniform in size and volume. Target and background DNA are distributed randomly into the droplets, meaning that each of them may contain no DNA sequence, background DNA, the target DNA sequence, or a combination of the previous ones. Modified from Droplet Digital™ applications guide, 2014 (Ref. 192, fig.1.3. and 1.4.).

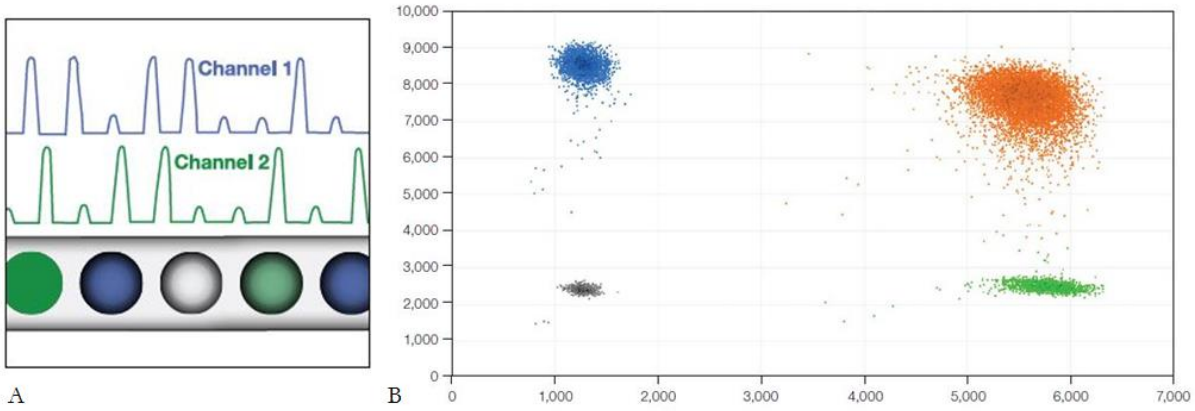


Figure 17. (A) Droplets are spaced out individually for fluorescence reading by the droplet reader. Positive droplets, which contain at least one copy of the target DNA molecule, exhibit increased fluorescence compared to negative droplets. **(B)** In a 2-D plot representing droplet fluorescence, droplets cluster into four groups: FAM-negative, HEX-negative (double negative droplets; bottom left-hand corner), FAM-positive, HEX-negative (droplets containing wild-type *KRAS*, bottom right-hand corner), FAM-negative, HEX-positive (droplets containing mutant *KRAS*, top left-hand corner), FAM-positive, HEX-positive (double-positive droplets). Modified from Droplet DigitalTM PCR Applications Guide, 2014 (Ref. 192, fig. 1.8. and 1.10.).

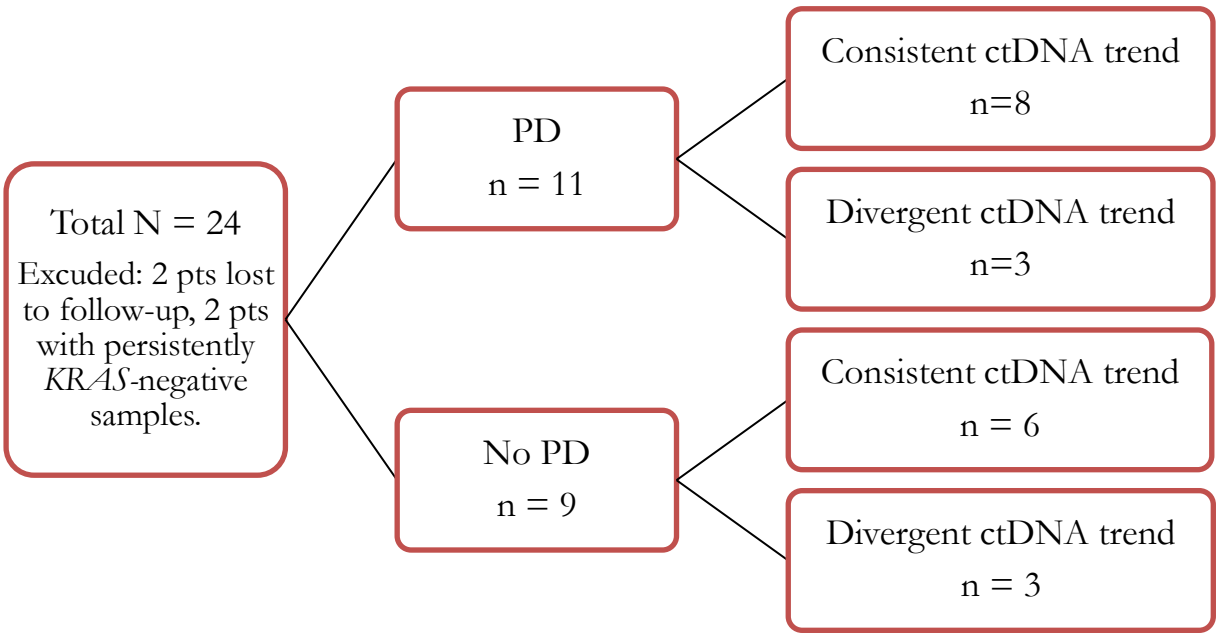


Figure 18. Circulating tumour DNA (ctDNA) trends for patients achieving disease control at the end of follow-up and patients encountering disease progression. A consistent trend was defined as an upward trend for patients experiencing progression of disease or a downward trend for patients with disease control. On the other hand, divergent trends were upward for patients with no evidence of progression at the end of follow-up, or downward for patients with radiological confirmation of progression during follow-up.

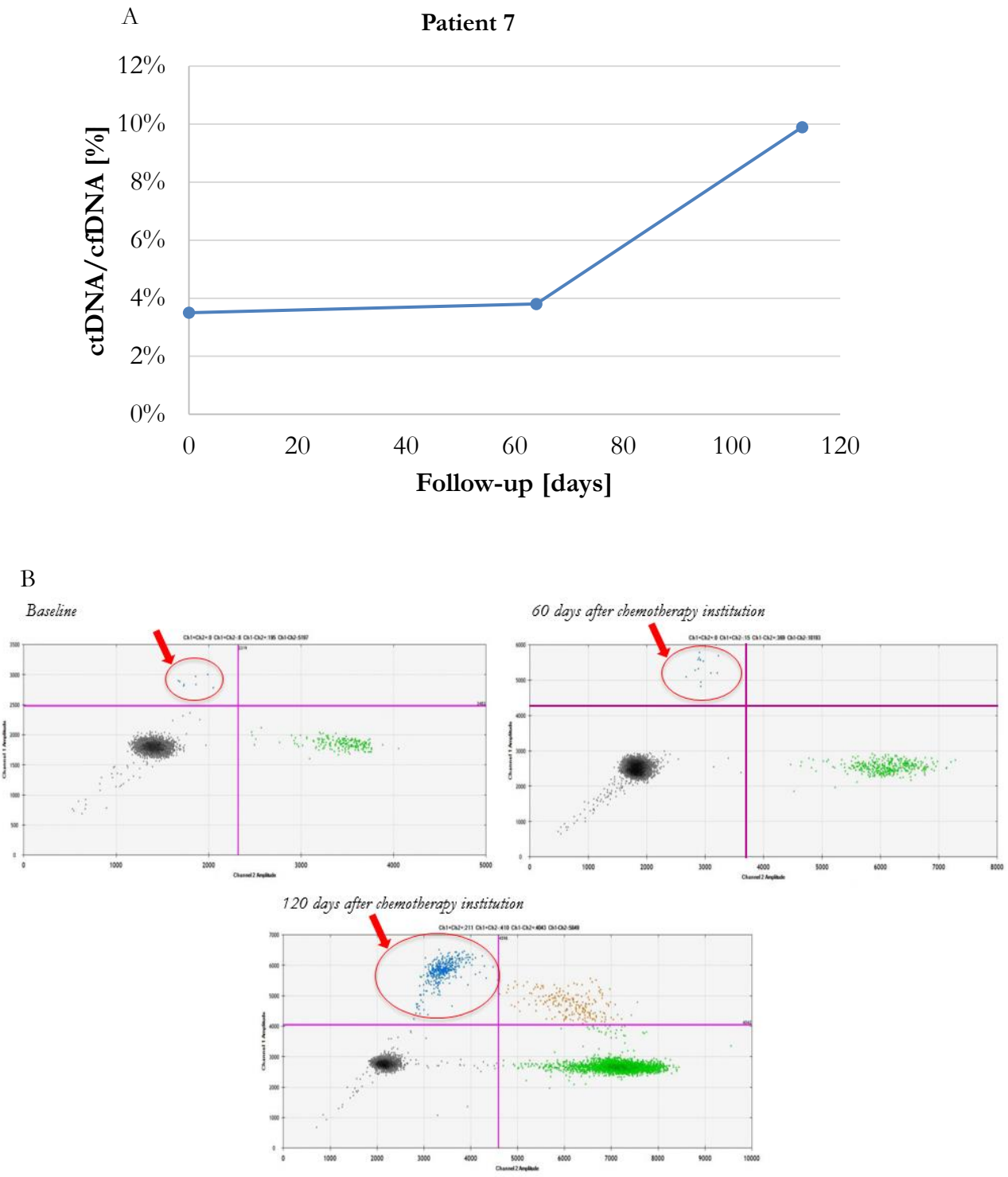
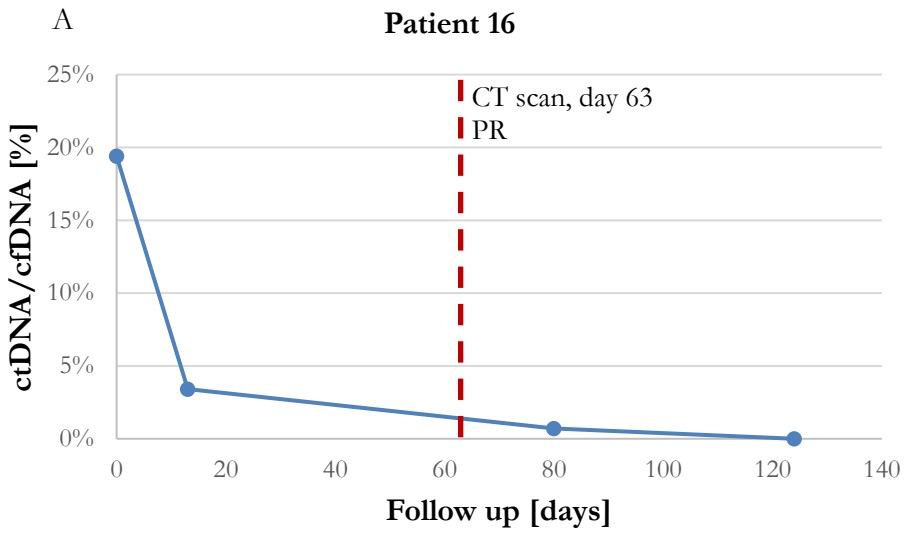


Figure 19. Circulating tumour DNA (ctDNA) trend in patient 7. **(A)** ctDNA levels gradually but continuously rise reaching a peak approximately at the same time as imaging confirms progression. cfDNA, circulating cell-free DNA, PD, progression of disease. CA19.9 levels are not shown because this patient did not have elevated CA19.9 concentrations. **(B)** The 2-D plots generated after ddPCR analysis illustrates that ctDNA levels are quite low at baseline, somewhat increased after 2 months of treatment and very high in the following follow-up sample.

A



B

CA 19.9	Date	Value
Baseline	02/03/2016	5592
Response	10/05/2016	4810

C

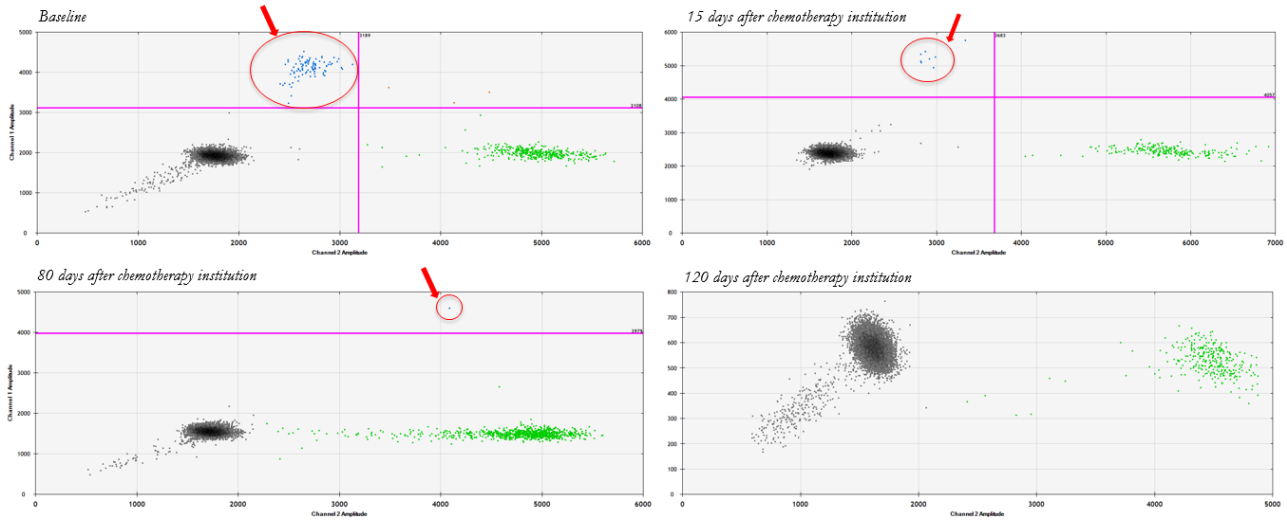


Figure 20. Circulating tumour DNA (ctDNA) and CA19.9 trends in patient 16. **(A)** A remarkable decline in ctDNA levels anticipated radiological confirmation of objective tumour response (PR, partial response) by more than 6 weeks. **(B)** The dynamic range of ctDNA was by far greater than that of CA19.9. **(C)** The 2-D plots generated by ddPCR clearly show how a relatively high baseline concentration of ctDNA dramatically declines in the subsequent follow-up samples, until no mutant *KRAS* droplets can be detected.

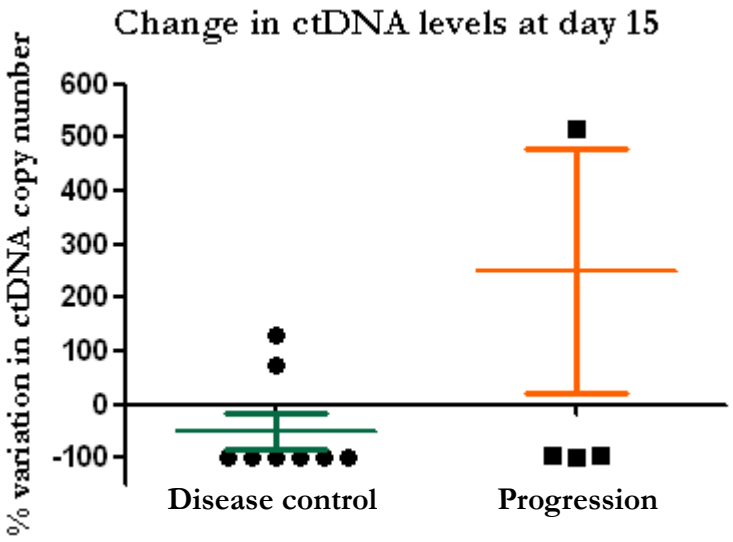


Figure 21. Patients who achieved disease control (left column) had ctDNA levels approximately halving (-48.8%) over the first chemotherapy cycle; patients who experienced progression had an average increase of 250.0% over the same time span. Error bars indicate the standard error of the mean. Statistical comparison, however, was non-significant ($p = 0.12$).

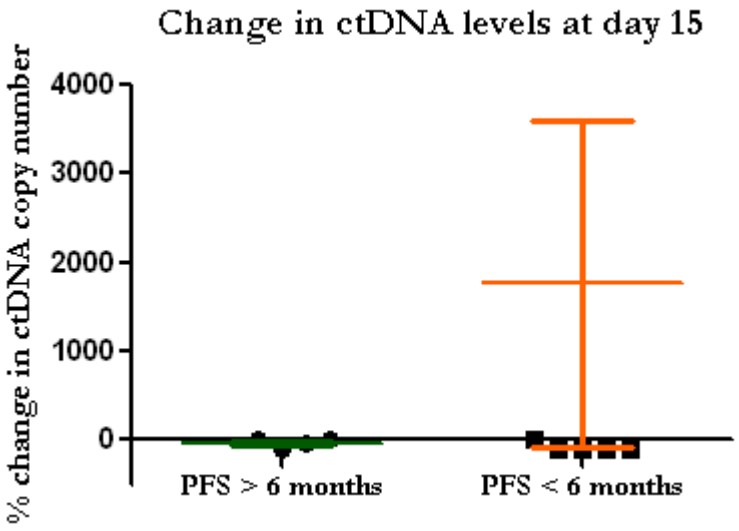


Figure 22. The graph shows that patients having a shorter PFS had, on average, a positive ctDNA variation index after 15 days from the initiation of chemotherapy, while patients with a longer PFS tended to have declining ctDNA levels. Error bars indicate the standard error of the mean, which is remarkably high for patients with a PFS shorter than 6 months. Statistical comparison was non-significant.

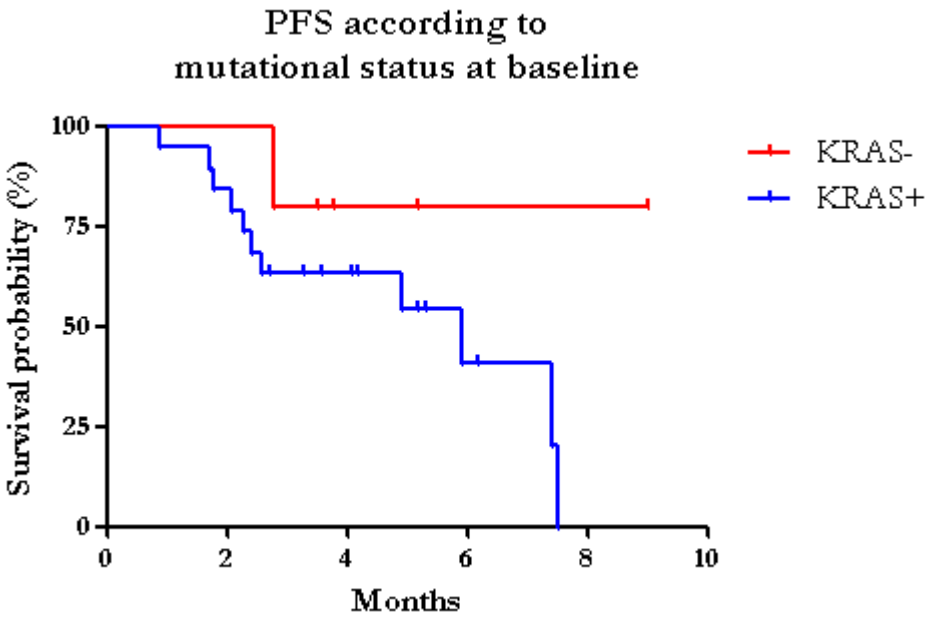


Figure 23. Kaplan-Meier curves for progression-free survival (PFS) in mutant *KRAS*-positive (KRAS+) and *KRAS*-negative (KRAS-) patients at baseline. Although the two curves clearly do not overlap (median PFS for KRAS+ patients, 5.9 months; median PFS for KRAS- patients, not reached), the sample size is too small to achieve statistical significance (HR 0,375, 95% CI 0.10 to 1.38, p = 0.14).

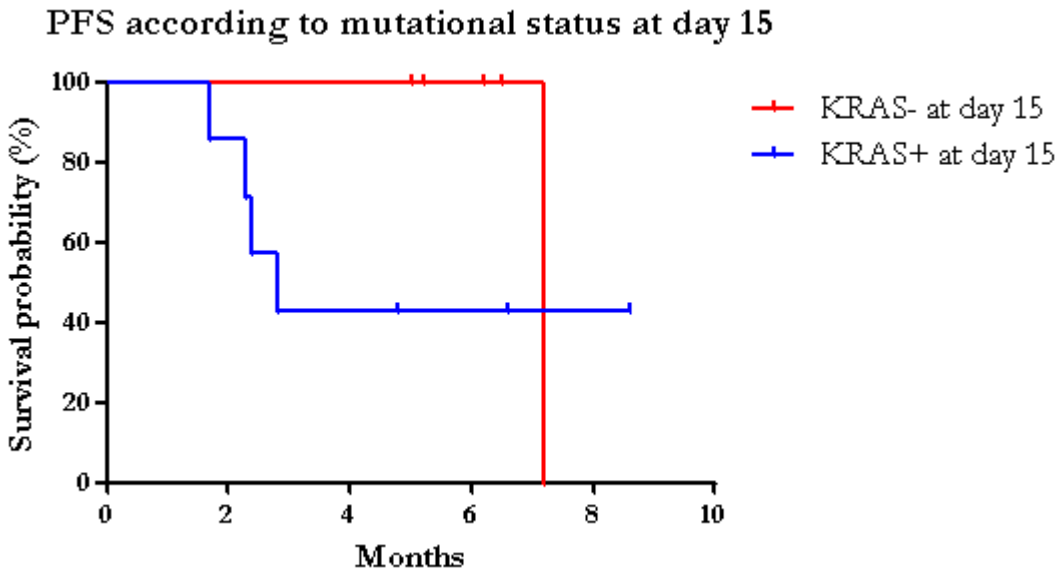


Figure 24. Kaplan-Meier curves for progression-free survival (PFS) in mutant *KRAS*-positive (KRAS+) and *KRAS*-negative (KRAS-) patients after one cycle of chemotherapy. Statistical analysis did not unveil a significant difference in PFS between the two groups (median PFS for KRAS- patients, 7.2 months; median PFS for KRAS+ patients, 2.8 months; p = 0.19).

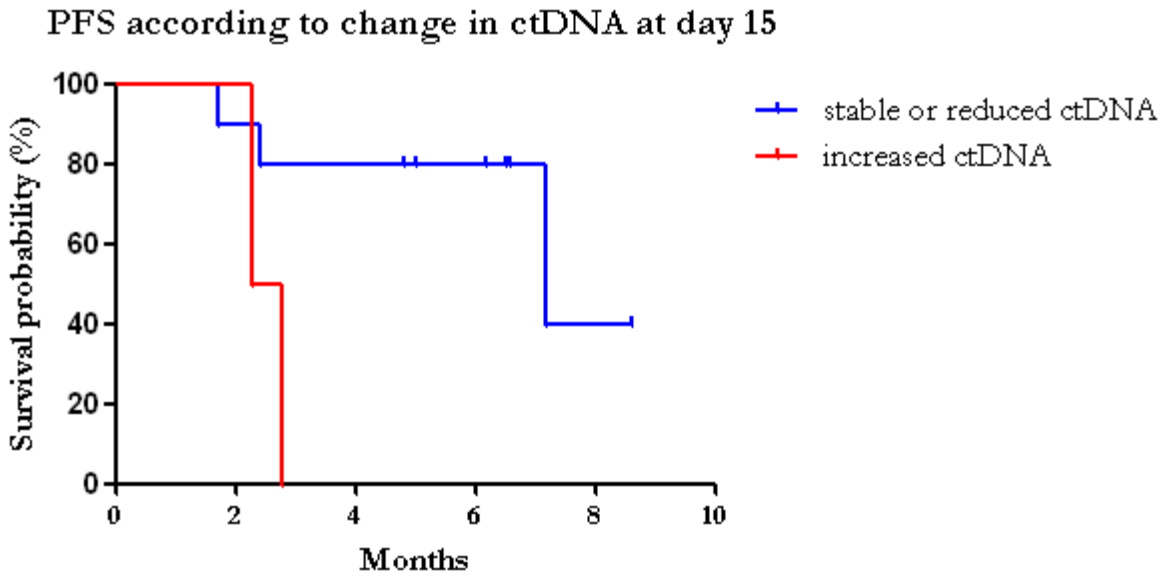


Figure 25. Kaplan-Meier curves for progression-free survival (PFS) in patients who had stable or declining ctDNA levels after one cycle of chemotherapy and subjects who showed an upward trend in ctDNA at the same time point. The median PFS for patients with stable or reduced ctDNA was 7.2 months, while it was 2.6 months for patients with increased ctDNA; statistical analysis unveiled a significant difference in PFS between the two groups (HR 0.05, 95% CI 0.01 to 0.83, p = 0.037).

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